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EFFECTS OF PREGANGLIONIC STIMULATION AND HEMICHOLINIUM NO. 3 ON CHOLINERGIC MECHANISMS IN A SYMPATHETIC GANGLION

BY

Ⓒ JAGDISH C. KHATTER

A THESIS
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Effects of Preganglionic Stimulation and Hemicholinium No. 3 on Cholinergic Mechanisms in a Sympathetic Ganglion, submitted by Jagdish C. Khatter in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date Jan 16, 1974

To my wife Brij and
children Kapil and Manishi

ABSTRACT

Preganglionic stimulation of the cat superior cervical ganglia at 60/sec for 2 to 8 min reduced the ganglionic acetylcholine(ACh) content by about 30%. With continued stimulation, the ACh stores gradually recovered within 15 min. However, when ganglia were allowed to rest following 4 min of stimulation at 60/sec not only was there a rapid restoration of ACh content, but the ACh levels rose to 130% of control after 10 min of rest. Under either of these experimental conditions the choline content increased transiently only after the ACh stores had returned to control values. The results are consistent with the concept that about one-third or more of the total ACh stores of a rested ganglion is in a form that can be readily mobilized for release. The observed rebound increase in the ACh content probably means that the ACh storage capacity is not normally saturable and that under most physiological conditions the ACh levels are maintained within certain limits by a precise control of ACh synthesis. Hemicholinium no.3 (HC-3) prevented not only the recovery of the ACh stores, but also the subsequent increase in both ACh and choline levels induced by 4 min of stimulation plus 10 min of rest. These latter results are in accord with the proposed mechanism of action of HC-3.

Investigation with HC-3 (1mg/Kg) has revealed that the nictitating membrane response to cholinergic nerve stimulation may be an unreliable index of the ability of HC-3 to inhibit ACh synthesis. The time-course effects of different doses of HC-3, in ganglia stimulated at 20/sec, indicate that 2 mg/Kg dose of HC-3 may be an optimal dose and appears capable of immediately and completely blocking ACh synthesis. When ganglia are pretreated with this dose of HC-3 and stimulated at 20/sec, a 50% re-

duction in the content of ACh occurs within 5 min. The data suggest that about 50% of the total ACh stores of a ganglion can be rapidly mobilized for release and the rest is only slowly converted to a releasable form.

That HC-3 can deplete ACh stores without reducing the choline content of ganglia, was also observed during the above investigations. The data indicate that HC-3 may not only block the uptake of extracellular choline but may also impair the intracellular utilization of choline for ACh synthesis.

When the above conditions of stimulation and HC-3 were applied to investigate ultrastructural changes, variable results were obtained. While in some cases the reduction in number of agranular vesicles appeared to correspond to the depletion in ACh content, in other cases no such relationship was evident. Also there was no increase in number of agranular vesicles corresponding to the rebound increase in ACh content. Under most conditions significant reduction in the density of agranular vesicles occurred mainly in the areas away from the synaptic region. These results do not necessarily negate the vesicle hypothesis and can be explained if one assumes that agranular vesicles stores variable amounts of ACh. The data also suggest that the vesicles from the non-synaptic zone may be mobilized to replace those depleted in the synaptic region during stimulation.

The number of large dark-core vesicles, of unknown content, also appeared to decline under the conditions which reduced the number of agranular vesicles. These observations indicate that the less numerous dark-core vesicles may also deplete their content during preganglionic stimulation. However, their content and the physiological significance of this observation is not known.

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HISTORICAL DEVELOPMENT

A. Evidence of Chemical Transmission

It is now almost a century since Du Bois-Reymond (1877) suggested that the motor nerves secreted a substance which activated the muscle. Lewandowsky (1898) and Langley (1901) noted independently the similarity between the effects of injection of extracts of adrenal glands and stimulation of synpathetic nerves. A few years later, in 1904, T.R. Elliot, while a student of Physiology at Cambridge, England, extended these observations and postulated that sympathetic nerve impulses release minute amounts of an epinephrine-like substance on immediate contact with effector cells.

Several years later, Dixon (1907) noticed the correspondence between the effect of muscarine and the response to vagal stimulation and in 1909 Dixon and Hamil advanced the hypothesis that the vagus nerve liberated a muscarine-like substance that acted as a chemical transmitter of its impulses. They suggested that excitation of nerve induces the local liberation of a hormone which causes specific activity by combination with some constituent of the end organ, muscle or gland. Dixon's theory met with universal skepticism which discouraged him from following this promising field of investigation.

In 1914 Dale made a thorough study of the pharmacological properties of acetylcholine (ACh) and some other choline esters. He observed that these drugs produced effects that were analogous to those observed after electrical stimulation of certain peripheral nerves. He was so impressed with the remarkable fidelity with which these drugs reproduced the responses to stimulation of parasympathetic nerves that he introduced the term parasympathomimetic to characterize their effects.

Loewi (1921) established the first real proof for chemical mediation of nerve impulses. In his ingenious experiment he stimulated the vagus nerve of a perfused (donor) frog heart and allowed the perfusion fluid to perfuse a second (recipient) frog heart used as a test object. The contraction of the second heart was inhibited by this fluid. With this experiment he demonstrated that a substance was liberated upon stimulation of the vagus trunk of the donor heart; this substance in turn inhibited the recipient heart producing an effect analogous to that of vagal stimulation. He called this substance "vagustoff". Loewi and coworkers subsequently reported that vagustoff had many properties in common with ACh (Loewi, 1932, 1933).

Many other investigators established quite conclusively that a chemical mediator is instrumental in the activity of all peripheral efferent nerves. The concept of chemical transmission of nerve impulses has been universally accepted at most synaptic junctions.

The evidence for chemical transmission of nerve impulses in sympathetic ganglia is discussed in the following section of the thesis.

B. Chemical Transmission in Sympathetic Ganglia

The evidence of chemical transmission in autonomic ganglia has been obtained chiefly from experiments on perfused ganglia. Kibjakow (1933) described a method for perfusing the superior cervical ganglion (SCG) of the cat with Locke's solution. The perfusate collected during electrical stimulation of the preganglionic nerve fibres was reinjected into the arterial supply of the SCG. A contraction of the nictitating membrane was observed similar to that caused by nerve stimulation. However, when the perfusate was collected in the absence of nerve stimulation no such contraction of the membrane was seen.

Feldberg and Gaddum (1934) repeated Kibjakow's experiment and found that the active substance could only be recovered in the perfusate if an anticholinesterase was present in the Locke's solution. They identified the active substance in the perfusate on the basis of its behavior or activity when subjected to five different pharmacological and biological tests. These observations supported the theory that the mechanism by which each nerve impulse normally passes the synapse consists in the liberation of small quantities of ACh. Feldberg and Vartianen (1935) observed that ACh appears in perfusion fluid after preganglionic but not antidromic stimulation. They also reported that physostigmine potentiates the effect of submaximal electrical stimulation as well as the ganglionic stimulant action of injected ACh. These findings further supported the chemical transmission theory in SCG.

Nicotine (Feldberg and Vartianen, 1935) and curare (Brown and Feldberg, 1936a) were shown to block transmission in the perfused SCG without impairing the release of ACh-like substance. Both of these blocking agents also prevented the effects of injected ACh on the ganglion. The results of these experiments added support to the contention that transmitter released on nerve stimulation in SCG is a choline ester.

The presence of enzyme for synthesizing ACh in SCG of cat was suggested by Brown and Feldberg in 1936b. They observed that the total amount of ACh liberated in stimulated ganglia perfused with choline-Locke's solution was several times of that obtainable from the contralateral unstimulated ganglion by extraction.

Bannister and Scrase (1950) used a relatively crude enzyme preparation derived from cat SCG and demonstrated that ACh can be synthesized *in vitro*. The enzyme preparations obtained from SCG, whose preganglionic nerve had been cut 10 days previously, were devoid of synthesizing activity. The above experiments indicated that the enzymes involved in ACh synthesis are located in the preganglionic nerve fibres.

More recently Friesen *et al.* (1965) demonstrated that labelled choline (^{14}C-choline), when added to the perfusion fluid, can be incorporated into ganglionic ACh and that labelled ACh can be liberated upon preganglionic stimulation. The ACh thus produced was identified as ^{14}C-ACh by paper electrophoresis and paper chromatography.

The sequence of events, which is now generally accepted, as taking place during the passage of an impulse across the synapse in sympathetic ganglia is the following: With the arrival of nerve impulse at the terminals of the preganglionic axon, the chemical transmitter ACh is released from storage sites in the nerve terminals; it diffuses across the narrow synaptic cleft (approximately 200 Å) and then generates a nerve impulse in the postsynaptic neuron (McLennan, 1963). Studies concerning the metabolism of ACh in SCG are more fully discussed in the next section.

C. ACh Turnover in SCG

As mentioned earlier the *in vivo* synthesis of ACh, in sympathetic ganglia of cat, was first suggested by Brown and Feldberg (1936). They observed that when the cat SCG were perfused with choline-free Locke's solution and stimulated preganglionically (up to 12/sec), the quantity of ACh liberated in the perfusate was much higher than the quantity originally present in the unstimulated ganglia. They also noted that the rate of ACh liberated was high at the beginning of stimulation and fell off progressively to reach a much lower level after 20 to 60 min of stimulation. This lower level of ACh release was then maintained with little further decline. Although one can assume that the fall in ACh output, after prolonged stimulation, was probably due to the lack of choline in the perfusion fluid, the stimulation did not change the extractable choline or ACh content of the ganglia. No explanation for the source of choline was provided.

Kahlson and MacIntosh (1939) confirmed the findings of Brown and Feldberg. They suggested that the stimulation (10/sec) did deplete the ACh depots, but that the deficit went unobserved by Brown and Feldberg because ACh was rapidly synthesized in the short interval between the removal of ganglion and its disintegration in the extracting medium. Kahlson and MacIntosh perfused the ganglia with Locke's solution containing glucose, mannose and pyruvate and suggested that these substances promote ACh synthesis. However, no choline was present in their perfusion fluid and no reference was made to the source of choline for this rapid synthesis of ACh. It is quite conceivable that inadequate synthesis of ACh, during prolonged stimulation, observed by these investigators might have been due to the unavailability of endogenous choline and/or lack of choline in the perfusion fluid.

In 1939 Rosenblueth *et al.* recorded the responses to submaximal doses of ACh (in SCG of cat) before and after prolonged preganglionic stimulation at 60/sec and noted that the threshold response of the SCG to ACh first decreases and later increases during prolonged preganglionic stimulation. In this connection they also observed that the ACh content of SCG first decreases and then increases during this period of stimulation. However, these latter findings were based on single experimental observations and no further work was carried out to validate or to study in more detail these changes in ACh content.

In 1953 Perry extended the work of Brown and Feldberg and studied the output of ACh during prolonged periods of preganglionic stimulation at different frequencies, namely; 5, 10, 20, 31 and 100/sec. The cat ganglia were perfused with Locke's solution with or without eserine and the output of ACh and choline were measured in the perfusate. In his studies, Perry observed that after 40 min of stimulation the total output of ACh is little influenced by varying the frequency between 5 and 100/sec. But the volley output falls more rapidly as the frequency of stimulation is increased (in the ganglia perfused with eserinized Locke's). Perry suggested that the amount of ACh liberated from ganglion by a single preganglionic volley is a constant fraction of the 'available' stock. He further hypothesized that only part of the ACh of a ganglion is readily available for release by nerve impulses and that while the ACh released by stimulation is quickly replenished by synthesis, the newly formed ACh only becomes available at a relatively slow rate, which is equal to the steady rate of ACh output (about 4 ng/min) after prolonged stimulation. However, in the investigations carried out by Perry, choline an important precursor of ACh was always absent from the perfusion fluid. This lack of choline may have influenced the rate of synthesis and thus the output of ACh in the perfusate. Furthermore, no investigations were carried out to study the influence of these different frequencies of stimulation on the ganglionic levels of ACh and choline. Source of choline for ACh output in perfused (with eserine Locke's) ganglia was also left unexplained in Perry's investigations.

Following up the observations (Schueler, 1955) that choline is an antidote for HC-3 poisoning (choline chloride, 100 mg/Kg intraperitoneally completely antagonizes 0.2 mg/Kg of HC-3 i.p. whereas 0.05 mg/Kg HC-3 was 100% fatal in a group of 50 mice), MacIntosh *et al.* (1956) tested the effect of HC-3 (2×10^{-5} M) on ACh output in plasma perfused SCG of cat. They perfused ganglia with plasma containing HC-3 and stimulated preganglionically at 20/sec. In their investigations it was found that after prolonged stimulation the rate of ACh release declined rapidly and at the same time ganglionic transmission gradually declined. When the ganglion was stimulated during perfusion with eserinized Locke's solution, instead of with plasma, it synthesized ACh much more slowly, and synthesis was still further reduced if HC-3 was present in the perfusion fluid. MacIntosh *et al.* (1956) suggested that HC-3 is a potent inhibitor of ACh synthesis, in SCG, and may compete with choline for a specific carrier system thus preventing its transport. (Work concerning the source of choline for ACh synthesis and the mechanism of action of HC-3 is discussed in the next two sections of this thesis.)

That ganglionic ACh was stored in different pools was suggested by Birks and MacIntosh in 1961. The mean value of ACh content per ganglion of 50 ganglia was found by these workers to be 266 ng. In their experiments when HC-3 (2×10^{-5} M) was added to Locke's solution, the perfused ganglion lost 81% of the ACh during one hour stimulation (20/sec). Birks and MacIntosh found that only 85% of the ganglionic ACh could be depleted by further stimulation and about 15% of total ACh could never be released by stimulation. They designated releasable ACh (85%) as depot ACh and the rest (15%) as extrasynaptic ACh which was not available for release by nerve impulses. Since, during prolonged stimulation, the initial output of ACh was always high (about 31 ± 2 ng/min) and fell off to a low value (4.3 ± 0.7 ng/min) in the last 15 min of one hour stimulation. Birks and MacIntosh suggested that depot ACh was made up of a smaller "readily-releasable" fraction and a larger fraction which serves as a reservoir from which the smaller pool is replenished. They further estimated that 23% (50 ng) of depot ACh (220 ng) of a ganglion was readily available fraction and the rest (170 ng) was only slowly available for release.

The data obtained in plasma perfused ganglia (Birks and MacIntosh, 1961) indicates that more ACh can be released during the first 5 min of stimulation at 60/sec than at 16/sec; thereafter, however, the rate of ACh release declines such that during later periods of stimulation the ACh liberated per minute is the same at both frequencies of stimulation. Birks and MacIntosh (1961) suggested that the two fractions of depot ACh were connected in series. The rate at which ACh can be discharged is limited by the rate of mobilization of ACh from the larger into the smaller, more readily releasable fraction.

The regulation of ACh synthesis was also investigated by Birks and MacIntosh (1961). They suggested that ganglia stimulated repetitively at frequencies up to 20/sec maintain their depot ACh at close to its resting level provided that they are supplied with the normal blood or plasma. Due to the constancy of this value of ACh, MacIntosh (1963) concluded that the amount of depot ACh is determined not merely by the balance between the conditions favouring synthesis and conditions favouring release, but primarily by the number of sites available for storage of ACh. According to this hypothesis, any excess ACh synthesized would then be immediately destroyed by intracellular cholinesterase.

Potter *et al.* in 1968 suggested that ACh synthesis may be regulated by mass action laws; that is, by the concentration of choline, acetylcoenzyme A, ACh and coenzyme A in the region of choline acetyltransferase. According to this proposal the main driving force for the synthesis of ACh would be the fall in concentration of ACh in the vicinity of choline acetyltransferase. Kaita and Goldberg (1969), however, have suggested that the regulation of ACh synthesis may be due to the feedback inhibition of the activity of choline acetyltransferase by accumulated ACh. In this regard they have shown that 10 mM to 100 mM concentrations of ACh reduce the activity of choline acetyltransferase by about 50%. However, these were only *in vitro* studies and whether or not this also occurs *in vivo* is still debatable.

D. Source of Choline

The fact that the endogenous choline may be available for ACh synthesis was first observed by Brown and Feldberg (1936). In their experiments when cat SCG was perfused with choline-free Locke's solution and stimulated, the quantity of ACh released during 60 min of stimulation exceeded the initial ganglionic ACh content. That ganglia may store choline was further supported by the work of Birks and MacIntosh (1961). They found that ganglia perfused with choline-free Locke's solution continue to discharge choline at the rate of 10 - 50 ng/min even in the absence of any stimulation.

In order to determine the source and levels of endogenous choline, Friesen *et al.* (1967) carried out chronic preganglionic denervation experiments. After 14 days of denervation of cat SCG, they found that only 30% of the total ganglionic content of choline was localized in the preganglionic nerve terminals. These observations lend additional support to the concept that a comparitively small amount of endogenous choline may be available for ACh synthesis in SCG. Since ACh synthesized by Locke-perfused ganglia exceeds their original stores (Brown and Feldberg, 1936), choline must be available from other sources to account for the synthesis of ACh in ganglia perfused with (eserinized) choline-free Locke's solution.

Choline associated with phospholipids may be available for ACh was suspected by Friesen *et al.* in 1967. They found that about 21 μg of choline is associated with phospholipids in SCG. However, most of this esterified choline is localized in postsynaptic structures rather than presynaptic terminals. The amount of choline released as a result of turnover of phospholipids and phosphorylchlorine was estimated more recently by Collier and Lang (1969). Their results indicate that during resting or stimulation only 4 ng/min choline was released from phospholipid turnover. This small amount of choline released from phospholipids may not be of significant value to a large amount of ACh synthesized in SCG. However, it may be utilized for a small steady output of ACh as observed by Perry (1953) after prolonged stimulation of ganglia perfused with (eserinized) choline-free Locke's solution.

Bligh in 1952 measured the choline levels of plasma and found that cat plasma contains about 5 x 10^{-6} M choline. It is noteworthy that the plasma choline concentration remains constant even after food or exercise. Birks and MacIntosh (1961) observed that addition of extra choline to the perfusing plasma does not appreciably increase the output of ACh during prolonged stimulation. They suggested that the level of choline in plasma may be enough for the optimal synthesis of ACh.

That extracellular choline is important in maintaining ACh stores in SCG was confirmed by Birks and MacIntosh (1961). They perfused ganglia with Locke's solution containing choline (3.5 x 10^{-5} M) and demonstrated that after 10 min of stimulation (at 20/sec) the steady levels of ACh obtained were significantly higher than in the experiments with choline-free Locke's solution. Even more striking was the effect of added choline on the extractable ACh content of stimulated ganglia. Instead of being reduced by 40-60%, as in the experiments with choline-free Locke's perfusion, the ACh content rose by 107%. They suggested that in the presence of choline, the ganglia are able to synthesize ACh at a rate similar to ganglia perfused with plasma. Birks and MacIntosh (1961) have estimated that synthesis of ACh within the ganglion (perfused with choline-containing fluid) is increased seven-fold during maximal preganglionic stimulation. Consequently the demand for choline must also be increased to ensure an adequate rate of synthesis of ACh. Since preganglionic terminals store only limited quantities of non-esterified choline (Friesen *et al.*, 1967) and little is apparently available from phospholipid sources, most of the choline for ACh synthesis during intense stimulation must come from an extracellular source.

More direct evidence for the utilization of extracellular choline in maintaining ACh stores in SCG was provided by Friesen *et al.* (1965). They demonstrated that radioactive choline (^{14}C-choline) added to the perfusion fluid can be incorporated in ACh stores of ganglia. More recently Collier and Lang (1969) have shown that the labelled choline present in the perfusion fluid can also be incorporated at a measurable rate into phosphorylcholine and phospholipids. It is thus clear that the level of extracellular choline is an important limiting source for ACh turnover during sustained activity in SCG, even though some ACh synthesis can be carried out in its absence (Brown and Feldberg, 1936).

Some of the extracellular choline trapped by ganglionic nerve terminals may have come from the hydrolyzed ACh liberated during stimulation. Perry (1953) observed that during preganglionic stimulation of non-eserinized choline-free Locke-perfused ganglia, the choline output first rose and then fell: the rise as he pointed out might be due to the appearance of hydrolyzed ACh and the fall to uptake of extracellular choline for ACh synthesis. He suggested that in the absence of eserine, choline derived from liberated ACh may be recaptured by the preganglionic nerve endings and used for ACh synthesis. More recently these observations were confirmed by Collier and MacIntosh (1969). Their experimental results indicate that about half of the choline obtained from released ACh can be recaptured by the preganglionic nerve terminals.

Ganglionic nerve endings indeed must be remarkably efficient in trapping extracellular choline. The performance is impressive when one recalls that choline does not readily penetrate into neurons (Koketsu *et al.*, 1959) and that the nerve endings account for less than 1% of the volume of the perfused ganglion (Birks and MacIntosh, 1963). Since HC-3 is thought to inhibit ACh synthesis by competing with choline for transport to the acetylation sites, it becomes a useful tool to investigate the metabolism of ACh in SCG. The detailed work concerning the mechanism of HC-3 is presented in the next section of this thesis.

E. Mode of Action of HC-3

It is now almost two decades since Long and Schueler (1954) synthesized a group of bis-quaternary ammonium compounds called hemicholiniums. Chemically they are bipheynyl choline derivatives cyclized by hemiacetal formation. The comprehensive studies by Schueler (1955) indicate that one of these compounds, hemicholinium no. 3 (HC-3), is very toxic to mice and has much lower anticholinesterase activity than its analogues. Its most striking pharmacological action is delayed respiratory paralysis and can be prevented by the prior administration of choline. On the basis of these and other findings, Schueler suggested that HC-3 acts by interfering with some cholinergic mechanisms.

MacIntosh *et al.* (1956) added (2×10^{-5} M) HC-3 in plasma perfusing cat SCG and found that, during prolonged stimulation, the rate of acetylcholine output rapidly declined and also ganglia lost most of their preformed ACh stores. This effect of HC-3 could be antagonized by raising the choline content of the perfusing plasma. When the ganglion was stimulated during perfusion with eserinized Locke's solution instead of plasma, it synthesized ACh much more slowly and synthesis was still further reduced if HC-3 was present. In their similar study on mouse brain (minced and incubated in eserinized Locke's), HC-3 (10^{-4} M) diminished the synthesis of ACh by 75%. Choline, when added, increased synthesis of ACh and partly (2×10^{-5} M) or wholly (10^{-3} M) reversed the inhibitory effect of HC-3. However, when HC-3 was added to an extract of acetone-dried rat brain powder in a medium supporting ACh synthesis, little or no inhibition of ACh synthesis was observed. MacIntosh *et al.* (1956) suggested that either HC-3 acts on intact nervous tissue by poisoning the enzyme choline acetyltransferase (which becomes less susceptible to HC-3 after being extracted from the tissue) or HC-3 compete with choline for transport to the intraneuronal sites of acetylation.

That HC-3 prevents ACh synthesis by interfering with choline transport was further supported by the work of MacIntosh *et al.* in 1958. They tested the effect of HC-3 on tubular excretion of choline by the avian kidney and found that 2 x 10^{-4} M HC-3, added to the infusion fluid, sharply diminishes the choline output. When similar tests were made on excretion of phenol red, no change in output was seen. Since avian renal tubule does not involve any acetylation of choline, MacIntosh *et al.* suggested that HC-3 interferes with acetylcholine synthesis in nervous tissue by competitively inhibiting the transport of choline to its intracellular site of acetylation.

Gardiner (1957) observed that acetylcholine synthesis by ether-treated guinea-pig brain powder was largely unaffected by HC-3. HC-3 (10^{-4} M) in the presence of 2.5 x 10^{-5} M choline reduced the amount of ACh by only 10 to 20%. However, in preparations untreated with ether, HC-3 (10^{-4} M) reduced the ACh formed in 4½ hr, by 84%. This further supported the hypothesis that choline acetylase itself is not affected by HC-3 but that the compound acts on the system transporting choline into the cell.

In 1959 Reitzel and Long studied the effect of HC-3 in rabbit using the sciatic-gastroconemius preparation. In their experiments, when the animal was injected (i.v.) with a low dose of HC-3 (100 μg/Kg) and the muscle preparation was stimulated at a frequency of 1/sec, a typical delayed block of neuromuscular conduction was observed. This blockade of neuromuscular conduction could be prevented by administration of 10 to 20 mg/Kg choline and reversed by rest. Reitzel and Long inferred that HC-3 was capable of acting presynaptically at the neuromuscular junction as it does at the sympathetic ganglion.

Thies and Brooks later in 1961 investigated the effect of large doses of HC-3 and found that HC-3 produces curare-like postsynaptic action at the guinea-pig neuromuscular junction. They determined muscle sensitivity to applied ACh, both in the presence and the absence of HC-3, by observing twitch threshold. In their experiments, Thies and Brooks found that within an hour after addition of 1.2 x 10^{-4} M HC-3 to the bathing medium, threshold concentrations of ACh required to produce twitches increased about 100 times. When 4 x 10^{-5} M eserine was added, HC-3 increased the concentration of ACh required to stimulate muscle fibres only 5 - 20 times instead of 100 times. Choline, however, was ineffective in reversing the action of this large dose of HC-3 at the neuromuscular junction. Thies and Brooks (1961) also found that HC-3 depresses the amplitude of spontaneous miniature end plate potentials, similar to curare, without independently changing their frequency and suggested that HC-3 may act postsynaptically at neuromuscular junction without affecting presynaptic release.

Birks and MacIntosh (1961) found that HC-3 (1 mg/Kg) caused about 70% depletion of ganglionic ACh when stimulated preganglionically at 20/sec for 20 min. A corresponding transmission failure was also observed after the first few minutes of stimulation. With post-ganglionic stimulation, on the other hand, a nearly maximal retraction of the nictitating membrane could still be maintained even after an hour of stimulation. Also the response to preganglionic stimulation, when it had failed, could be partially restored either by rest, or by lowering the frequency of stimulation or by injecting choline. The degree of restoration with choline depended on the choline:HC-3 ratio. When the molar ratio was 5:1 or 50:1, the inhibitory effect was still present; when it was 1000:1, the normal ACh synthesis was restored and the ganglia gained ACh while they were being stimulated. Birks and MacIntosh (1961) concluded that HC-3 acts in ganglion presynaptically by inhibiting ACh synthesis.

Peripheral and post-synaptic effects of HC-3, thus, were unequivocally demonstrated, but the evidence for a presynaptic effect at the neuromuscular junction were still indirect.

The studies of Elmquist, Quastel and Thesleff (1963) provided the first direct evidence that HC-3 could reduce the transmitter output from motor nerve endings. Their study was made in the isolated rat phrenic diaphragm preparation and it was found that in the presence of 2×10^{-6} M HC-3, a concentration of the drug which had little curare-like effect, prolonged stimulation of the nerve produced a progressive decrease in transmitter quantum size corresponding to the progressive increase in blockade of neuromuscular transmission.

The early failures to obtain neuromuscular block upon stimulation of a nerve-muscle preparation probably reflected the very slow rate of stimulation that had been used, and many of the perplexing findings reported by various investigators could be reconciled in the light of HC-3's proven ability not only to interfere with synthesis of transmitter, but to block immediately, in higher doses, the normal reaction of ACh with end plate receptors. However, the observations of Elmquist *et al.* (1963) indicate that the resting of the nerve-muscle preparation after its run down by prior stimulation, in the presence of HC-3, was only followed by a partial recovery of the quantum size. Several hours seem to be required for the recovery to be appreciable in the rat-diaphragm preparation. Peculiarly, choline was largely without effect in antagonizing the ability of HC-3 to decrease quantum size *in vitro*. Not only that, the addition of choline to the bathing medium while the preparation was being rested, led to only a slightly faster recovery of the unit size as compared to rest alone.

Triethylcholine, known to interfere with ACh synthesis in nervous tissue (Burgen *et al.*, 1956) behaves essentially like HC-3 at the frog neuromuscular junction. Bowman *et al.* (1961) have shown that triethylcholine has curare-like as well as prejunctional action. Matthews (1966) studied the effect of HC-3 and triethylcholine on ACh output in plasma-perfused ganglia of the cat and found that despite their almost identical inhibiting effect on ACh synthesis, the rate of recovery, when perfused with plasma containing excess choline, differs with these two substances. Recovery from inhibition by triethylcholine was more rapid than the corresponding reversal of HC-3 inhibition. More recently Sellinger *et al.* (1969) have studied the intracellular distribution of ^{14}C-HC-3 in canine caudate nucleus and found that ^{14}C-HC-3 was associated in the fraction containing mitochondria-filled nerve endings. The nerve endings containing fewer mitochondria showed much lower affinity for HC-3. They suggested that HC-3 does not remain on the cell surface but penetrates into the nerve endings.

This entrance of HC-3 into presynaptic structures and the previous findings indicating slow recovery of HC-3 inhibition by excess choline suggests that action of the drug is not merely to compete with choline at the outer membrane surface but may have one or more sites of action within the nerve terminals. One of these actions may be the inhibition of ACh synthesis by competing with choline at some membrane site within the nerve terminals. It is also possible that ACh is synthesized in the nerve ending cytoplasm and then transported into synaptic vesicles from which it may be released; HC-3 could inhibit this transfer of ACh.

Thus it would be of interest to investigate what effect HC-3 has on the endogenous levels of choline (and ACh) of SCG under different conditions of stimulation.

F. Synaptic Vesicles and ACh Storage in Cholinergic Nerve Endings

Fatt and Katz, in 1952, observed spontaneous discharges of amplitude of the order 1/100 of the normal end plate potential, in the functional region of the isolated resting frog nerve-muscle preparation. Except for their spontaneous random occurrence and their small size, the discharges are indistinguishable from the end plate potentials produced by nerve impulses. These discharges, so-called miniature end plate potentials, were found to be greatly reduced in size by a small dose of curare (5×10^{-7} M) and increased by a small dose of prostigmine. Fatt and Katz suggested that miniature epp's arise from localized impacts of small individual units of ACh upon the post-synaptic membrane.

Del Castillo and Katz (1954) recorded epp's in isolated frog nerve-muscle preparation using intracellular recording technique. They observed that by lowering the Ca^{++} concentration and increasing the Mg^{++} concentration, the amplitude of the epp can be reduced to that of spontaneous miniature potential. They further analyzed statistically the fluctuations in amplitudes of recorded epp's and suggested that the epp is built up of small all-or-none quanta which are identical in size and shape with the spontaneously occurring miniature potentials. The number of units making up the epp (number of quanta) decreases when the magnesium concentration in the bathing solution is raised. Raising the calcium concentration causes an increase in number of quanta.

Further evidence for the quantal concept was provided by the work of Del Castillo and Katz in 1956. By applying small doses of ACh to the end plate, by ionophoresis, they observed that the size of depolarization is graded according to the dose of ACh, and its time course varies with the distance and speed of application. Since the potentials evoked by one or a few molecules of ACh are far below the resolving power of the recording method they concluded that discrete potentials changes like the miniature epp's, with their regular size and time course, can only arise from a synchronous action of packets of ACh containing a large number of molecules at a time.

Boyd and Martin (1956) and Liley (1956) obtained similar results in mammalian (cat) nerve-muscle preparation and suggested that quantum phenomenon observed by Del Castillo and Katz may be universal. Liley (1956) and Katz and Miledi (1965) further studied the curarized mammalian end plate during stimulation of the motor nerve terminals and found that the size of the ACh packet is not altered by the membrane change associated with the nerve impulse. Instead it alters the frequency or the probability of occurrence of the quantal event. Thus they concluded that the size of the ACh quantum remains constant in spite of widely varying conditions of cell membrane from which it is released.

About the same time the ensuing search for a morphological correlation of the transmitter quanta concluded that the neuromuscular junction and presumably other chemically transmitting synapses were characterized by small spherical bodies, the so-called synaptic vesicles (De Robertis and Bennett, 1955) adjacent to the synaptic cleft on the presynaptic side. De Robertis and Bennett studied earthworm nerve-muscle preparation and frog sympathetic ganglia and found that in both cases pre- and post-synaptic membranes (about 70 to 100 Å thick) were separated by a gap of about 100 to 150 Å. Small synaptic vesicles, about 200 to 500 Å in diameter, were present in only the presynaptic side. In subsequent years similar observations were made by other investigators in different preparations, e.g., in frog neuromuscular junction (Palay, 1956; Robertson, 1956; Birks *et al.*, 1960), cat ganglia (Taxi, 1957; Grillo and Palay, 1962), rabbit ganglia (Causey and Hoffman, 1956) and frog ganglia (Taxi, 1961). The temptation to equate quantal release with the vesicle packaging was presumably almost irresistable (De Robertis and Bennett, 1955; Del Castillo and Katz, 1955, 1956). The so-called vesicle hypothesis of synaptic transmission thus implies that quanta of transmitter are stored and released in association with the presynaptic vesicles. Random and scattered release at rest produces miniature potentials and simultaneous release of large numbers of quanta, due to the nerve impulse, produces the post-synaptic potential.

In an attempt to test the morphological aspect of the vesicle hypothesis, Liley in 1956 and later Birks *et al.* (1960) studied the ultrastructure changes on denervation of the frog neuromuscular junction. Birks *et al.* (1960), by recording intracellularly, found that epp's as well as vesicles disappear after 5 to 10 days of nerve degeneration. This provided further support to the association of vesicles with the spontaneous release of ACh quanta.

Alterations in vesicle number accompanying various experimental procedures of physiological interest have also been investigated. Rapela and Covian (1954) reported that on stimulation of splanchnic nerve in dog, secretion of catecholamines occurs maximally at frequencies from 40 to 100/sec. They also noticed a considerable reduction in output when stimulated at high frequency. De Robertis and Ferreira (1956) studied ultrastructural changes in rabbits under similar conditions of stimulation. They found that on stimulation of splanchnic nerve at 100/sec for 10 min, the mean number of synaptic vesicles increased from $82.6/\mu^2$ to 132 vesicles per μ^2 of nerve ending area. Similarly, when stimulated at 400/sec for 10 min, the number of vesicles decreased to $29.2/\mu^2$. De Robertis and Ferreira suggested that destruction and formation of vesicles in the nerve endings is a continuous process. When stimulated at 100/sec both destruction and formation of vesicles are accelerated but due to the increase in catecholamine output (Rapela and Covian, 1954), the increase in vesicle formation dominates. However, when stimulated at 400/sec, the formation process cannot keep pace with the destruction due to the fatigue occurring at this frequency and thus corresponds to low output of catecholamine shown by Rapela and Covian (1954).

However, attempts to produce similar changes in vesicle number of frog neuromuscular changes were unsuccessful (Birks, Huxley and Katz, 1960). Birks *et al.* noticed an uneven distribution of vesicles, in the nerve endings, with a tendency of accumulation near the synaptic cleft. However, no significant change in vesicle number was detected on stimulation by these workers.

The main experimental support for the vesicle hypothesis comes from the analysis of ACh distribution within intact nerve endings (Whittaker *et al.*, 1964) isolated from homogenized guinea-pig brain cortex. Whittaker *et al.* (1964) isolated synaptic vesicles by suspending the synaptosomes in distilled water and submitting the suspension to density gradient separation. About 50% of ACh remained bound to the structures, mainly synaptic vesicles, and was not accessible to hydrolysis by cholinesterases. However, the remaining 50% of synaptosomal ACh could not be accounted for by vesicles or by vesicle hypothesis of transmitter release. Whittaker *et al.* (1964) suggested that this was an osmotically labile fraction of ACh which may be located in synaptosomal cytoplasmic sap and can be preserved with cholinesterase inhibitors.

The drug hemicholinium no. 3 (HC-3) has been shown to reduce the amount of ACh synthesized within nerve terminals (MacIntosh, Birks and Sastary, 1956; Gardiner, 1957), presumably by competing with choline at some stage leading to its acetylation to form ACh. In search for morphological changes, Csillik and Joö (1967) studied the effect of HC-3 on ultrastructure of parietal cortex and caudate nucleus of rat brain. They found that when rats were injected with HC-3 (10 mg/Kg) I.P. and killed after 23 - 35 min of injection, a significant decrease in vesicles occurred in the parietal cortex, but no change in vesicle number was observed in the caudate nucleus. However, the control vesicle number, as determined by Csillik and Joö (1967), were significantly higher in the parietal cortex than in the caudate nucleus. Furthermore, the decrease of vesicle number, in the presence of HC-3, was only to the level of vesicle population in caudate nucleus. These variations may be due to structural changes during the interval the tissue was isolated and subjected to fixation or due to difference in amount of HC-3 reaching the tissue.

Hubbard and Kwanbunbumpen in 1968 investigated the effect of ACh release with KCl on the vesicle population of neuromuscular junction in the rat diaphragm. When nerve-muscle preparations were soaked in 20 mM KCl for two hours, a significant depletion in vesicle population close to the synaptic membrane occurred. Under the same conditions Parsons *et al.* (1965) found that the number of available quanta is significantly increased. Similarly, depolarizing currents applied to the nerve terminals were found to reduce the available stores of ACh (Hubbard and Willis, 1968) yet they increased the number of vesicles close to presynaptic membrane (Landon and Kwanbunbumpen, 1968).

Although several other conflicting evidences are available, both in favour and against the hypothesis, data relating to the quantitative aspects of ACh content and release with morphological changes at junctional sites are rare. Formidable technical difficulties lie in the way of any attempt to find significant alterations in vesicle populations after procedures which affect ACh stores. For example, in most cases the tissue has to be isolated, after treatment, before fixation. This interval between isolation and fixation may cause alteration in transmitter levels which may cause corresponding repair or reformation of depleted vesicles and thus change the vesicle population.

Some of the problems can be overcome by the use of superior cervical sympathetic ganglia (SCG) as a preparation for the investigation of vesicle phenomenon. SCG can be fixed while still being stimulated and a paired control ganglion is also available to study the corresponding changes in the transmitter levels. Thus present investigations were undertaken, using cat SCG, to study the correlation, if any, between the vesicle population and quantitative changes in ACh content under different experimental conditions.

RATIONALE FOR PRESENT RESEARCH

A. Acetylcholine Content and the Storage Capacity of Superior Cervical Sympathetic Ganglia of Cats

The present study arose from the chance observation that the ganglia resting for about an hour following several minutes of stimulation, at 60/sec, appeared to contain more ACh than is normally stored in the unstimulated ganglia of cat. The survey of the literature revealed that a similar preliminary observation had been made by Rosenblueth *et al.* in 1939. These workers found that the ACh content of a superior cervical sympathetic ganglion of the cat first decreases and then increases during prolonged period of stimulation at 60/sec. However, these latter findings were based on single experimental observations and no further attempts were made by these workers to rigorously substantiate this transient change in ACh content of ganglia during stimulation at 60/sec. Also, these observations appeared to be in conflict with the proposal made by MacIntosh in 1963.

MacIntosh (1963) observed that the ganglionic content of ACh remains relatively constant during rest or preganglionic stimulation at 20/sec. Evidently these ganglia possess the ability to rapidly increase the rate of synthesis thereby quickly replacing the ACh released during excitation. In this regard MacIntosh (1963) proposed that the ganglia store no more than a fixed quota of ACh because during rest or activity the ACh binding sites are nearly saturated; any ACh synthesized in excess of that which can be stored would be hydrolyzed by acetylcholinesterases within the nerve terminals. However, this proposal does not explain the changes in ACh content observed when these ganglia are stimulated at frequencies greater than 20/sec (Rosenblueth *et al.*, 1939). These preliminary observations indicate that the storage capacity for ACh is normally not saturable or additional sites can be produced.

With the objectives in mind to validate the observations of Rosenblueth *et al.* and to clarify the above conflicting evidences, the time-course effects of preganglionic stimulation at 60/sec on the content of ACh and choline of the superior cervical sympathetic ganglia of cat was studied in the present thesis. The influence of rest periods on ganglionic stores of ACh and choline after initial stimulation at 60/sec, was also investigated.

B. The Time-Course Effects of Hemicholinium no. 3 on the Acetylcholine and Choline Stores and the Estimation of the Size of Readily Releasable Pool of Acetylcholine in Sympathetic Ganglia of the Cat

Our investigations on 60/sec stimulation revealed that about 30% of the ACh content of ganglia can be depleted within 2 min of stimulation. This observation can be interpreted to mean that one-third or more of the total ACh stores of a rested ganglion can be mobilized for release within 2 min; for one must assume that some of the ACh liberated during this stimulation period was replaced by resynthesis. How much of the readily mobilizable pool of ACh is immediately available for release is a debatable issue and may be difficult to determine with any degree of accuracy. However, Birks and MacIntosh (1961) have proposed that about 50 ng (23%) of ACh in a resting ganglion may be ready for immediate release. This compares with about 90 - 120 ng (30 - 40%) of ACh per ganglion, which can be estimated to be in the immediately releasable form based on our results during 60/sec stimulation. These latter results were of sufficient interest to warrant further research in this area.

The drug HC-3 has been shown to reduce the amount of ACh synthesized within the nerve terminals (MacIntosh *et al.*, 1956; Gardiner, 1957) presumably by competing with choline for transmport to acetylation site. However, very little attention has been paid to the time-course effects of HC-3 on ACh or choline content of nerve tissues. With the previous results in mind it was felt that useful information regarding the size of the readily releasable pool of ACh, and choline metabolism in sympathetic ganglia, may be obtained by further investigation in this area. Specifically, we decided to investigate the time-course of the effects of different doses of HC-3 on ACh and choline content in ganglia stimulated at different frequencies of stimulation.

C. Ultrastructure of Presynaptic Nerve Endings in Relation to the Ganglionic Stores of Acetylcholine

The discovery of the quantal nature of ACh release (Fatt and Katz, 1952; Del Castillo and Katz, 1954) and the subsequent finding that the nerve endings contain numerous agranular vesicles, lead to the suggestion that these vesicles were involved in the storage and release of ACh (De Robertis and Bennett, 1955; Robertson, 1966). Somewhat later Whittaker and co-workers (Whittaker *et al.*, 1964) were able to demonstrate that agranular vesicles isolated from brain tissue contain appreciable quantities of ACh. Attempts have also been made to test the morphological aspects of the vesicle hypothesis. Thus, some authors have reported a decreased number of vesicles during increased transmitter release (De Robertis and Vaz Ferreira, 1957; Hubbard and Kwanbunbumpen, 1968), others have reported an increased number (De Robertis and Vaz Ferreira, 1957) and still others have not observed any changes (Birks, Huxley and Katz, 1960; Burnstock and Merrilees, 1964). One most important aspect would be to search for the morphological changes in terminals, particularly the vesicle population, during experimental conditions which cause quantitative changes in the transmitter stores.

Our previous results indicated that ganglia release more than 30% of the ACh stores, within a few minutes, if stimulated at 60/sec. The data further suggested that this lost ACh content can be recovered very quickly if the ganglia are allowed to rest after the initial stimulation at 60/sec. The data on the effects of stimulation, in the presence of HC-3 (2 mg/Kg), revealed that about 50% of the ACh stores of ganglia can be released within a few minutes. These results prompted us to investigate if similar conditions of stimulation, HC-3 and the rest periods following initial stimulation, would cause alterations in the number of agranular vesicles corresponding to the changes in ACh content of ganglia.

Thus the major objective of this aspect of the research was to establish what relationship, if any, exists between the ACh content of the superior cervical ganglion of the cat and the number of vesicles in the preganglionic nerve terminals. In this regard the ultrastructural changes in the presynaptic nerve endings were determined in relation to:

(a) ACh content of ganglia during preganglionic stimulation at different frequencies.

(b) ACh content of ganglia during preganglionic stimulation at 60/sec, followed by different rest periods.

(c) Preganglionic stimulation at different frequencies in the presence of HC-3.

MATERIALS AND METHODS

A. Drugs and Other Solutions

Some of the drug solutions in the present investigation were unstable in aqueous solution; hence it was frequently necessary to prepare fresh solutions of these agents.

1. α-chloralose

α-chloralose solution 80 mg/ml was prepared just before use by dissolving the drug in propylene glycol, preheated to 80°C in a water bath.

2. Acetylcholine stock solution

The stock solution of acetylcholine (2 μg/ml) was prepared by dissolving acetylcholine iodide in 0.9% saline containing 0.2% acetic acid and was stored in a freezer. It is noteworthy that use of acetylcholine iodide is made because it is less delequescent and more stable than acetylcholine chloride. Fresh stock solution was prepared about every month. Dilutions from the stock solution were made just prior to each bioassay.

3. Hemicholinium no. 3 solution

Different concentrations of hemicholinium bromide were prepared in 0.9% saline solution and stored in the freezer. Fresh stock solutions were prepared about every two weeks.

4. Locke's solution

The Locke's solution used for bioassay had the following composition in g/l: NaCl, 7.5 g; KCl, 0.42 g; $CaCl_2.2H_2O$, 0.32 g; $NaHCO_3$, 2.1 g; and dextrose, 1.00 g. The solution was aerated for 30 min with oxygen.

5. Millonig's buffer

Millonig's buffer was prepared by titrating 40 ml of 2.26% monobasic sodium phosphate ($NaH_2PO_4.H_2O$) solution to pH 7.3 with 5N NaOH. Five ml of 10.8% glucose was then added and the total volume was adjusted to 50 ml using distilled water.

6. Fixative solution

The fixative (2% glutaraldehyde) was freshly prepared by mixing 0.2 parts of 50% glutaraldehyde solution with two parts of the above prepared Millonig's buffer. The desired osmolarity (equivalent to 0.9% saline) was then obtained by diluting this solution with 2.8 parts of distilled water. Before the fixative solution was utilized, it was filtered through sintered glass.

B. Surgical Procedures

Cats were anesthetized with α-chloralose 80 mg/Kg administered intraperitoneally. The trachea and one femoral vein were cannulated. In experiments where it was necessary to record blood pressure, one femoral artery was also cannulated. Superior cervical ganglia along with the sympathetic trunks and carotid arteries were exposed. Whenever necessary the lingual and external carotid arteries were also exposed for ligation. Most of the arterial and venous branches of the carotid artery and jugular vein were ligated. A loose ligature was placed around each postganglionic trunk to facilitate rapid removal of ganglia. The preganglionic trunks of both ganglia were ligated and cut.

C. Stimulation Parameters

Warm mineral oil was placed in the neck cavity and surrounded the preganglionic nerve trunk of the SCG. The nerve trunk on one side was stimulated with a bipolar platinum electrode and the contralateral ganglion served as a resting control. In experiments with HC-3, the drug was administered I.V. 5 min prior to the beginning of stimulation. Pulses of 2 msec duration, at different frequencies, were delivered by a Grass Model SD5 stimulator. The animals were grounded during stimulation. A supramaximal voltage (usually 8 v) was employed in all of the above experiments. Stimulation response was judged by the degree of mydriasis and/or the isometric contractile response of the nictitating membrane. During prolonged period of stimulation the electrode was moved forward every 5 to 10 minutes. Approximately a minute before the end of the experiment the loose ligature around the postganglionic trunk was tied in place. The ganglia were removed within 15 sec by approaching them from the postganglionic end and the last thing cut was the preganglionic stump. This procedure ensured that the ganglia were being stimulated until the last possible moment.

D. Extraction Procedure

Several procedures have been used for the extraction of ACh and choline from the tissue. However, the values of ACh obtained by our procedure (hot ethanol extraction) are similar to those reported by other investigators who have extracted with 10% trichloroacetic acid (Birks and MacIntosh, 1961; Matthews, 1966). Also ethanol can be removed by simple evaporation whereas the removal of trichloroacetic acid is a more time consuming procedure. In addition, traces of TCA may adversely affect the bioassay preparation.

Excised ganglia were immediately immersed in a 95% ethanol solution at 80°C containing 0.2% acetic acid, and maintained at this temperature for 10 min. The ganglia were then minced and heated for an additional 5 min at 80°C to ensure the complete extraction of ACh and choline. The final volume of the extract was 4 ml per ganglion.

E. Bioassay Procedure

An aliquot of the ganglionic extract was evaporated at 40°C, with the aid of a stream of nitrogen gas, to remove all the ethanol. The resultant residue was then dissolved in 0.8% NaCl and bioassayed for ACh with the use of guinea-pig ileum preparation (MacIntosh and Perry, 1950). Another aliquot was evaporated for the estimation of choline and was then acetylated by the method of Emmelin and MacIntosh (1956) using acetylchloride, and then bioassayed for ACh activity.

A number of tests were performed to verify that the biological activity measured was due to ACh (Chang and Gaddum, 1933; MacIntosh and Perry, 1950). The assay values obtained with the ileum preparation were not significantly different from those determined by the vasodepressor response in cats. Atropine blocked the biological activity in both preparations and the incubation of the solutions in whole blood at room temperature for two min destroyed the ACh-like activity. Similarly when samples were heated in alkaline solution of pH 11 for 1 min at 100°C, their ACh-like activity was lost. In contrast no appreciable loss of activity was obtained by heating samples for 1 min at 100°C in a solution of pH 4.

F. Measurements of Choline in Plasma

To determine the concentration of choline in cat plasma, about 1½ ml of blood was withdrawn from the femoral vein into a syringe containing 3 units of heparin. The contents of the syringe were immediately put into centrifuge tubes which in turn were placed in an ice bath. The cellular elements were removed by centrifugation at 4°C; ½ ml of plasma thus obtained was mixed with 3½ ml of hot (80°C) 95% ethanol containing 0.2% acetic acid. This mixture was heated for 10 min at 80°C and the precipitated proteins removed by centrifugation. Aliquots of this ethanol solution were then evaporated and acetylated by methods already described in bioassay procedures for the estimation of ganglionic choline.

G. Procedures Involving Ultrastructure Studies

1. Fixation of ganglia

To ensure a rapid and uniform fixation of all parts of the tissue, ganglia were perfused through the carotid artery with the aid of an infusion pump. Fixation by perfusion is considered superior to flooding the tissue externally, for the latter method will take a much longer time to diffuse into the tissue (Maunsbach *et al.*, 1962). About a minute before the end of the experiment the lingual and the external carotid arteries were ligated. The main carotid artery was then rapidly cannulated and the ganglia were perfused for about 10 min with a 2% solution of glutaraldehyde prepared in Millonig's buffer (Millonig, 1961). To minimize the dilution, the tissue was perfused with excessive fixative for the first few seconds and perfusion was then maintained at the rate of 1.6 ml/min. At the end of the perfusion period ganglia were excised and placed in the glutaraldehyde solution. The main body of the ganglia were cut longitudinally into three pieces and were usually stored in the fixative solution at 5°C for several days. Since most of the presynaptic nerve endings are localized in the central area, the middle section was generally selected for subsequent electron microscopic studies.

2. Post-fixation with osmium tetroxide

Before embedding, the tissue was post-fixed with osmium tetroxide. Osmium tetroxide, being a strong oxidizing agent, is readily reduced by the unsaturated lipid components of tissue and forms osmium blacks along with osmium oxide as a by-product. Because of its electron scattering properties the atoms of co-ordination compounds of osmium remaining in the tissue contribute to the formation of contrast in electronmicrographs.

In order to minimize the possible reaction between glutaraldehyde and osmium tetroxide during post-fixation, most of the excess fixative was washed off. The tissue was rinsed with 1 ml of Millonig's buffer for about 15 min; this procedure was repeated three times. After rinsing, the tissue was removed and plunged into a test tube containing 1% (w/v) osmium tetroxide. The tissue was kept in contact with osmium tetroxide for about an hour or until it turned dark brown.

3. Embedding

After post-fixation of the tissue with osmium tetroxide, the tissue was washed once again with Millonig's buffer to remove any excess of fixative. The tissue was then subjected to dehydration with graded concentrations of ethanol (from 50% to absolute). The aim of dehydration is to remove all the free water from the fixed and washed tissue and to replace it with a suitable organic solvent such as ethanol. According to Millonig (1966), ethanol causes less contraction of cellular materials than most other known solvents.

After dehydration with the final concentration of ethanol (absolute ethanol), the tissue was kept in contact with one ml of propylene oxide for about 15 min. Because most resins are not readily miscible with ethanol, propylene oxide is used as a transitional solvent before embedding in the epoxy resin. It should be noted, however, that propylene oxide is very reactive even at low temperatures and should not be used for longer than 15 min. If allowed to penetrate for a longer period, it may combine with the reactive groups in the cells and may affect certain histochemical and staining reactions.

The tissue was then embedded in epon 812 using standard molds. Epon 812 resin, because of low viscosity, penetrates into the tissue specimen faster than other resins and can be easily hardened uniformly at low temperatures with the addition of acid anhydrides and an amine accelerator. In the present investigation DDSA (dodecenyl succinic anhydride) and NMA (nadic methyl anhydride) were the acid anhydrides used. DMP-30 [2,4,6-tri(dimethylaminomethyl)-phenol] was used as an accelerator.

The following embedding formulation, originally reported by Luft (1961), was used in our investigations:

Mixture A	Mixture B
Epon 812 - 66 ml	Epon 812 - 100 ml
DDSA - 100 ml	NMA - 84 ml

Final embedding mixture:

Mixture A - 5 ml
Mixture B - 5 ml
DMP-30 - 0.1 ml (1%)

Since the molecular weight and viscosity of all the ingredients differ, it is imperative to mix all the ingredients very thoroughly. The accelerator should be measured carefully, otherwise the block becomes dark in color and too brittle for satisfactory sectioning.

The tissue samples were kept in embedding medium at room temperature for 4 to 6 hr followed by 72 hr at 60°C.

4. Sectioning

After embedding, the samples were sectioned into 60 to 100 mμ thick slices, using a MT-2 Ultramicrotome. The thickness of a section was estimated by observing the interference color in light reflected from the section while it is floating on the surface of distilled water in the trough. After sectioning, the sample sections were collected on copper grids with the help of camel hair.

5. Staining

Generally, the tissue is stained more than once in order to achieve adequate differential electron opacity. In the present investigation, the tissue was stained with lead citrate and post-stained with uranyl acetate.

The tissue sections were treated with saturated solution of lead citrate for about 15 min. The excess of the solution was then washed and the sections dried with filter paper. This was followed by staining with uranyl acetate. After staining with uranyl acetate for about 15 min the sections were once again washed and dried on filter paper. Since bound or free fixative in the tissue may react with the stain, every effort should be made to wash out the unbound fixative before staining.

6. Preparation of electron micrographs

A model JEM-7A (Japan Electron Optics Laboratory Co. Ltd.) electron microscope was used for preparing electron micrographs. Five to seven pictures per sample were taken at a magnification of 25,000. The main criteria used for the identification of the nerve endings was the specialized contact between pre- and post-synaptic membranes, visualized as a dark region separating the two membranes. A picture was taken each time such a nerve ending was seen with the electron microscope. These electron micrographs were then magnified three times during printing. Thus the final magnification obtained in photographs was 75,000. To eliminate bias, neither the person taking the electron microscopy pictures nor the person assessing the ultrastructural changes was aware of the experimental conditions to which the ganglia under investigation had been subjected.

7. Evaluation of electron micrographs

The population of vesicles, either agranular or dark-core, in preganglionic nerve terminals is expressed as the number of vesicles per μ^2. To obtain these figures, the total number of the respective vesicles in the nerve endings was counted and the total area of the nerve endings as depicted on the electron micrographs was determined in μ^2, using a planimeter. No corrections were made for the areas occupied by mitochondria or other vesicular bodies (e.g., area occupied by dark-core vesicles while evaluating changes in number of agranular vesicles) while determining the number of vesicles per μ^2.

In order to estimate whether the distribution of vesicles clustered near the presynaptic membrane was affected by the experimental procedures, a line was drawn on the electron micrographs parallel to this membrane at a distance of 2000 Å from it. A perpendicular line was drawn at each end of the synaptic junction; 2 regions, non-synaptic area and a zone of 2000 Å near the pre-synaptic membrane were thus defined. The concentration of the vesicles in both of these regions was evaluated. The statistical significance of the effects of the experimental procedures on vesicle concentration was tested by Dunnett's test of analysis of variance (Dunnett, 1955).

RESULTS

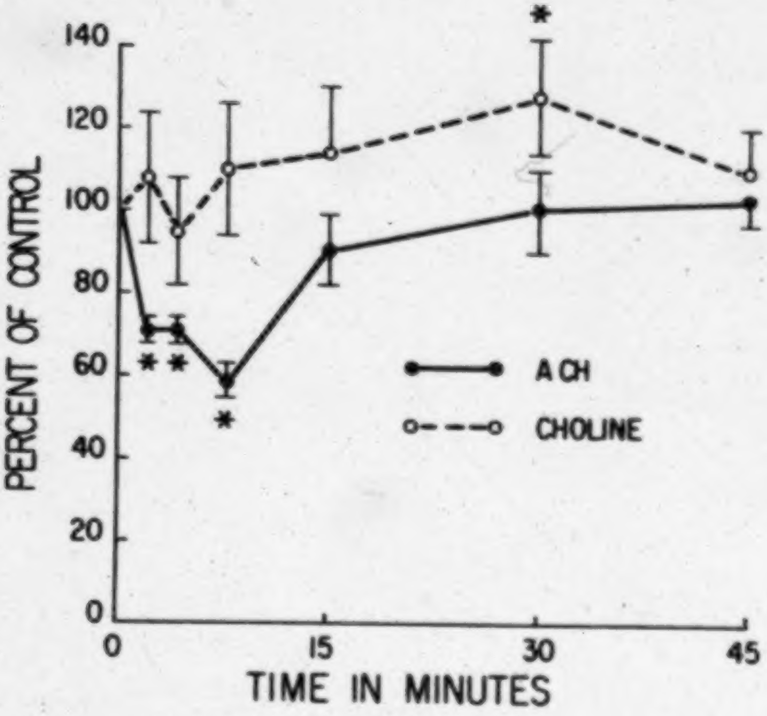
A. Acetylcholine and Choline Content of Control Resting Ganglia

The ACh and choline content (mean ± S.E.) of 111 control resting ganglia were 280 ± 7.2 ng and 243 ± 9.2 ng per ganglion respectively. The values of ACh obtained with the hot ethanol extraction procedure are similar to those reported by other investigators who have extracted with 10% trichloroacetic acid (Birks and MacIntosh, 1961; Matthews, 1966). Ganglionic choline levels varied considerably from cat to cat ranging from 94 to 593 ng per ganglion; this may explain why the mean value 243 ng is somewhat higher than the 173 ng per ganglion (average of 19 ganglia) reported earlier by Friesen *et al.* (1967). However, the choline content of ganglia from the same cat is relatively constant. In six experiments where the resting ganglia from the same cat were compared, the individual choline values for the corresponding left and right ganglia did not differ by more than 15% and the mean values varied less than 2%.

B. Effects of Preganglionic Stimulation on Acetylcholine and Choline Content of Ganglia

The time-course effects of continuous preganglionic stimulation at 60/sec are illustrated in Fig. 1. The superior cervical ganglia in all experiments were maintained *in situ* by their own intact blood supply. The ACh stores were reduced by 30 - 40% within the first 8 min and then they gradually returned to control values after a total of 15 - 30 min of continuous stimulation. Ganglia stimulated for 45 min contained the same amount of ACh as the contralateral unstimulated ganglia.

Fig. 1. The Effects of Continuous Preganglionic Stimulation at 60/sec on the ACh and Choline Content of Cat Superior Cervical Ganglia



The effects of stimulation at 60/sec for 2 - 45 min are depicted. The data are expressed as a percentage of the values obtained in the contralateral resting ganglia, and each point represents the mean ± S.E. of four to seven experiments. Asterisks indicate $p < 0.05$ in comparison with the resting controls. Solid line (0—0) represents ACh whereas broken line (0--0) represents choline content

Choline levels, in contrast, did not change significantly during the first few minutes of stimulation but thereafter began to increase to a maximum of about 130% after 30 min of stimulation. Subsequently the choline content declined and approached control levels at 45 min.

In view of the results obtained with a frequency of 60/sec, experiments were conducted to determine whether a similar decrease in the ACh content could be induced by stimulation at lower frequency. The data presented in Table I demonstrate that ganglia stimulated at 20/sec for 2 or 8 min maintained their ACh stores.

Experiments were also conducted to determine whether stimulation at 60/sec could cause a reduction in the ACh content of ganglia which have been previously stimulated at lower frequency. The data are presented in Table II. Although stimulation of previously rested ganglia at 60/sec decreased the ACh content, this frequency of stimulation was unable to produce such a change in ganglia stimulated for 30 min at 20/sec just prior to increasing the frequency to 60/sec. Probably the best explanation for these results is that the size of readily releasable pool of ACh was reduced during the period of stimulation at 20/sec.

Birks and MacIntosh (1961) have proposed that a normal resting ganglia contains about 50 ng of ACh in the form which is immediately available for liberation. This estimate may be too low, since preganglionic stimulation at 60/sec for 2 min decreases the ACh content by 90 ng; this amounts to about 30% of the total stores of ACh in the ganglion. However, in order to make an accurate estimate of the size of this pool on the basis of a reduction in the ACh content, one would have to know not only the amount of preformed ACh which was mobilized into a releasable form, but also the quantity of the recently synthesized ACh re-

TABLE I

Table I illustrates the inability of preganglionic stimulation at 20/sec for 2 and 8 min to lower the ganglionic ACh content. For the purpose of comparison, the effects of stimulation at 60/sec for the same duration are also presented. The values represent mean ± S.E. of 4 to 7 experiments and are expressed as ng/ganglion.

Stimulation Parameters	ACh Content (ng per ganglion) Control Resting	ACh Content (ng per ganglion) Stimulated	% Change in ACh Content
2 min 20/sec	314 ± 37	319 ± 13	+1.6
2 min 60/sec	310 ± 28	221 ± 18	-28.8*
8 min 20/sec	253 ± 22	269 ± 35	+6.3
8 min 60/sec	313 ± 32	184 ± 15	-41.2*

* $p < 0.05$ as compared to the resting contralateral control ganglion.

TABLE II

Comparison of the ability of an 8 min period of ganglionic stimulation at 60/sec to decrease the ganglionic ACh content of previously resting ganglia, with the effects seen in ganglia which have been stimulated at 20/sec for 30 min just prior to switching the frequency to 60/sec. The influence of stimulation for 30 min at 20/sec is also presented. The values represent the mean ± S.E. of 4 - 6 experiments and are expressed as ng/ganglion.

Stimulation Parameters	ACh Content (ng per ganglion)		% Change in ACh Content
	Control Resting	Stimulated	
8 min 60/sec	313 ± 32	184 ± 15	-41.2*
30 min 20/sec & 8 min 60/sec	271 ± 33	301 ± 34	+11.1*
30 min 20/sec	309 ± 17	359 ± 26	+16.2*

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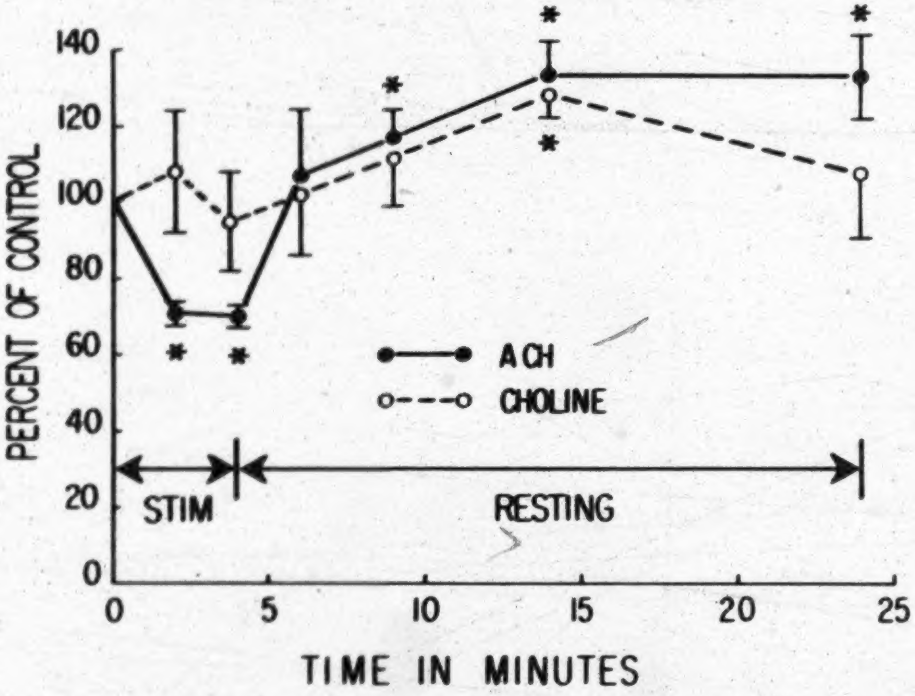
* $p < 0.05$ as compared to the resting contralateral control ganglion.

tained by the ganglion during 2 min period of stimulation.

C. Rate of Recovery of the ACh Content During a Resting Period After Preganglionc Stimulation

With continuous stimulation at 60/sec the ganglia eventually recover the ACh lost during the initial period of stimulation, but this process occurs quite slowly over a period of about 15 min. Hence it was decided to determine how quickly ganglia would restore the ACh when allowed to rest following 4 min of stimulation at 60/sec. The results are illustrated in Fig. 2. In these experiments the ganglia were stimulated at 60/sec for 4 min and then were allowed to rest for 2 to 20 min. The results show that under these conditions the ACh stores recovered within 2 min; but thereafter the ACh content continued to rise and reached 130% of control after 10 min of rest. During the initial 2 min of rest these ganglia synthesized at least 45 ng/min; this rate is considerably higher than the 29 ng/min observed by Birks and MacIntosh (1961) in plasma perfused ganglia stimulated at 20/sec. These data indicate that ganglia can very quickly resynthesize any ACh lost during stimulation and clearly demonstrate that this tissue can store more than a fixed quota of ACh even in the absence of an anticholinesterase drug. The choline content also rose during the rest period and attained 128% of the control values after 10 min of rest. Subsequently choline levels declined toward control levels. In this experiment as well as in the one depicted in Fig. 1, the choline levels increased only after the ACh content had returned to control values. This rise in choline content was transient in both cases suggesting that the excess was

Fig. 2. Rate of Recovery of ACh Content from 4 min of Stimulation at 60/sec During a Subsequent Rest Period



The effects of preganglionic stimulation at 60/sec for 4 min followed by 2 - 20 min of rest on the ACh and choline content of cat superior cervical ganglia. The data are expressed as a percentage of the values obtained in the unstimulated contralateral ganglia. Each point represents the mean ± S.E. of 4 to 7 experiments. Asterisks indicate $p < 0.05$ in comparison with the controls. Solid line (0—0) represents ACh and broken line (0--0) represents the choline content.

either removed from the ganglion by some process or converted to other choline derivatives within this tissue.

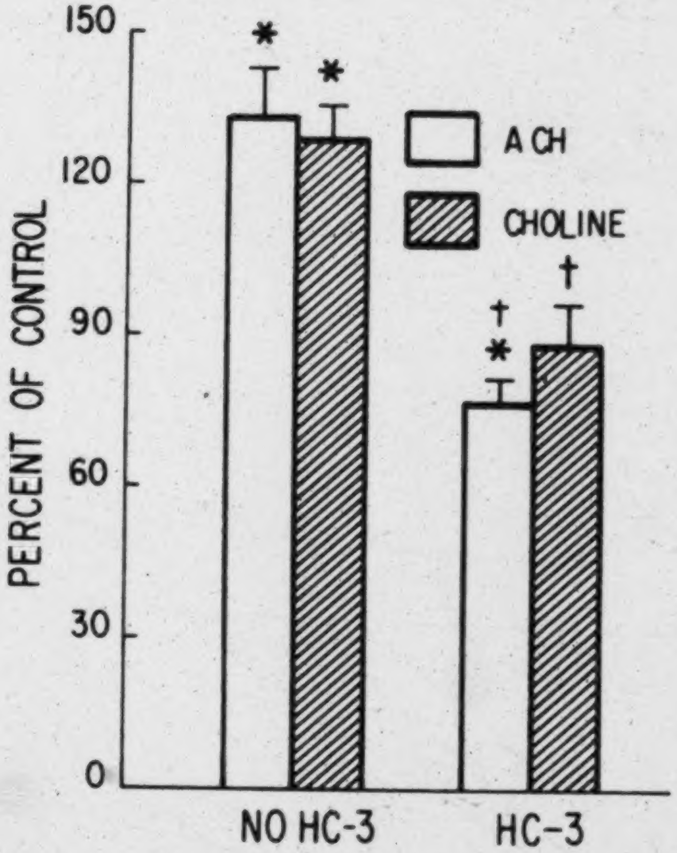
When HC-3, 1 mg/Kg, was administered intravenously 5 min prior to the onset of stimulation, the drug prevented the increase in both ACh and choline previously observed (Fig. 3) during the rest period. The results obtained with HC-3 are consistent with the proposal that the drug inhibits ACh synthesis by blocking the transport of choline to the sites of acetylation (MacIntosh *et al.*, 1958).

Experiments were also designed to ascertain whether frequencies lower than 60/sec followed by a rest period could cause alterations in ACh levels of ganglia. Under the conditions chosen, stimulation for 4 min at 5/sec or 20/sec plus 10 min of rest failed to induce statistically significant increases in either ACh (Table III) or choline. However, the data do indicate a progression in ACh content as the stimulation frequency is increased from 5 to 20/sec and suggests that, particularly in the latter situation, ideal conditions for demonstrating major changes may not have been chosen.

D. Effects of Hemicholinium no. 3 on Acetylcholine and Choline Levels in Ganglia Stimulated at Different Frequencies

A search of the literature revealed that the time course effects of HC-3 and the influence of different frequencies of stimulation, had not been thoroughly examined in terms of tissue levels of ACh and choline. In particular we were interested in investigating a correlation, if any, between ACh and choline content and the ability of this drug to cause transmission failure in stimulated sympathetic ganglia. It was also

Fig. 3. Effects of Hemicholinium No.3 (1 mg/Kg) on the ACh and Choline Content of Cat Superior Cervical Sympathetic Ganglia Stimulated at 60/Sec for 4 Min Followed by Rest



The bar graphs illustrate the ability of HC-3 to prevent the recovery in ACh and choline content during 10 min of rest after stimulation at 60/sec for 4 min. The data are expressed as a percentage of the values obtained in the unstimulated contralateral ganglia and represents the mean ± S.E. of four to seven experiments. Asterisks indicate $p < 0.05$ in comparison with the unstimulated controls, daggers indicate $p < 0.05$ in comparison with values obtained in stimulated ganglia not exposed to HC-3.

TABLE III

The effects of preganglionic stimulation at different frequencies for 4 min followed by 10 min of rest on the ganglionic ACh content. The values given represent the mean ± S.E. of 5 to 6 experiments and are expressed as ng/ganglion.

Stimulation Frequency	Acetylcholine Content Control	 Stimulation + Rest	 % Change
5/sec	291 ± 32	316 ± 56	+8.6
20/sec	268 ± 15	313 ± 34	+16.8
60/sec	307 ± 40	409 ± 83	+31.2*

* $p < 0.05$ as compared to the unstimulated contralateral ganglion.

hoped that this research will provide further insight into the mechanism of action of HC-3 in cholinergic transmission.

The effects of HC-3 (1 mg/Kg) on the content of ACh and choline of ganglia stimulated at different frequencies for 30 min are illustrated in Fig. 4. Although the maximum depletion (50%) of ACh was observed in ganglia stimulated at 10 and 20 pulses per sec, the magnitude of the reduction was largely independent of the rate of stimulation from 2 to 20 pulses per sec after the 30 min period of stimulation. These results are in sharp contrast to those seen on monitoring the nictitating membrane response (Fig. 5). Here, the rate of onset and degree of transmission failure appeared to be directly related to the frequency of stimulation. Thus at stimulus rates of 1 to 5/sec little evidence of impaired ganglionic transmission was observed, and the contractile response declined more rapidly in ganglia stimulated at 20/sec than those stimulated at 10/sec. None of the decreases in choline content (Fig. 4) were statistically significant.

In order to explore the relationship between ACh content and the degree of transmission further, the time-course effects of HC-3 (1 mg/Kg) in ganglia stimulated at 20/sec for 5 to 30 min were investigated. The results are summarized in Fig. 6. It is particularly noteworthy that HC-3 did not cause any decrease in ACh levels during the first 5 min of stimulation. Thereafter, however, the ACh content rapidly declined to 50% of control values at 10 min and remained at this level for the remainder of the stimulation period. A comparison of these results with the corresponding nictitating membrane response (bottom tracing in Fig. 5) indicates some correlation between the onset of transmission failure at 20/sec and the time-course of the effects of

Fig. 4. Effects of HC-3 (1 mg/Kg) on the ACh and Choline Levels of Ganglia Stimulated for 30 Min at Different Frequencies

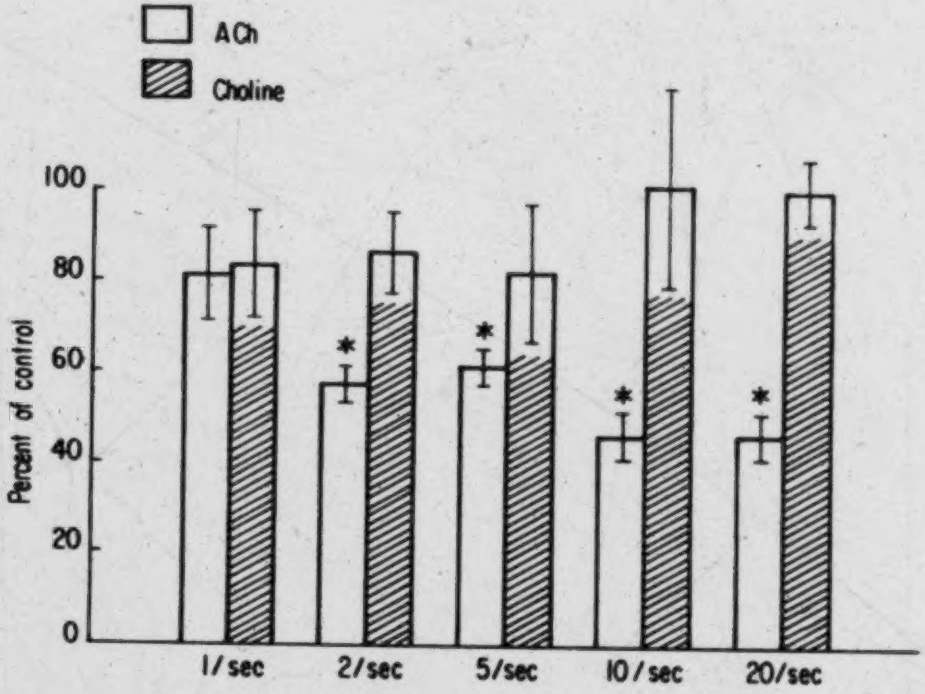
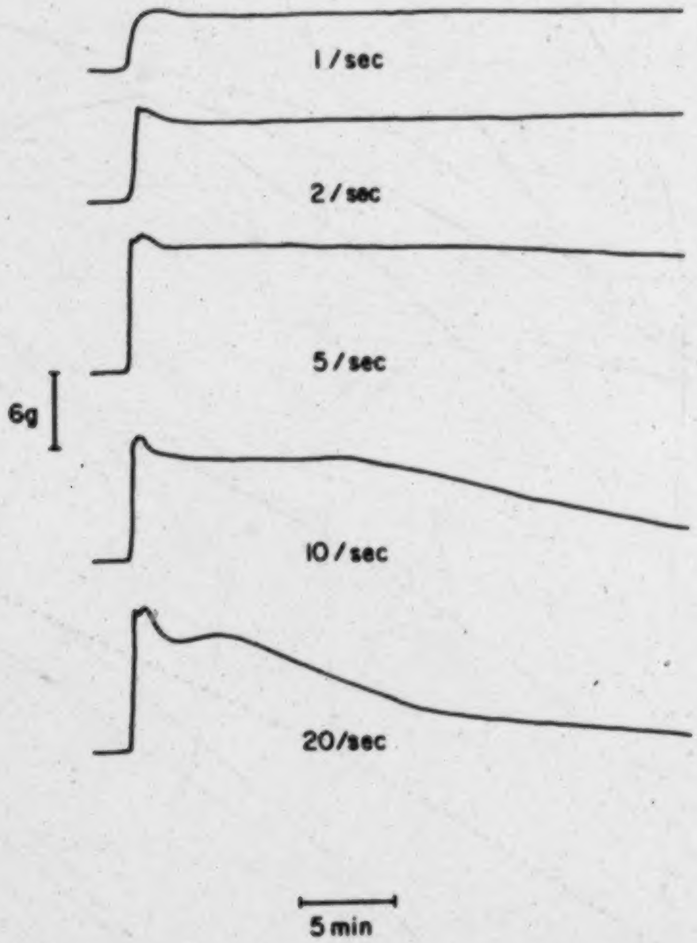


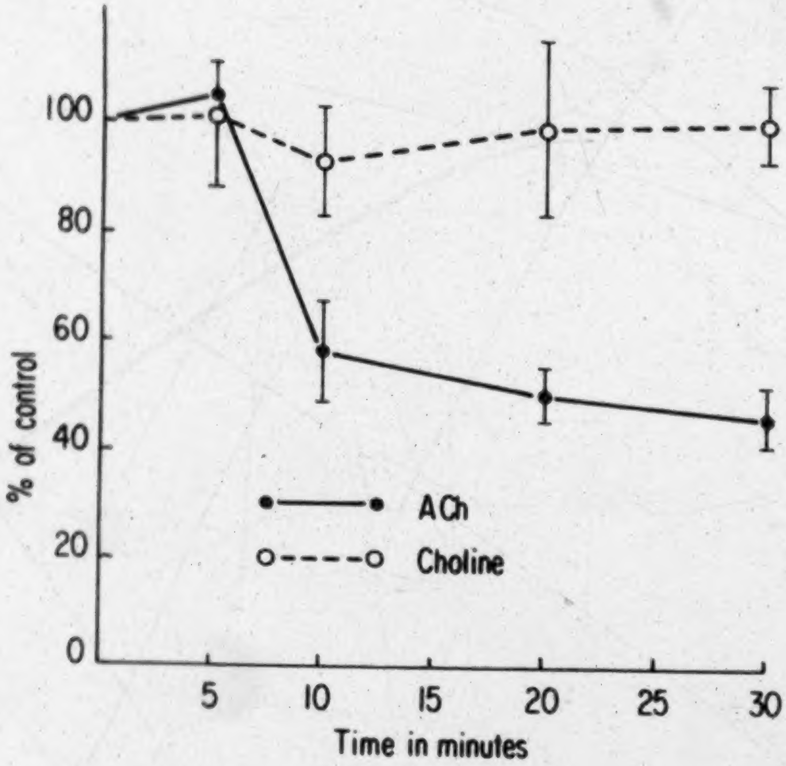
Fig. 4 illustrates the effects of preganglionic stimulation for 30 min at different frequencies on the ganglionic content of ACh and choline. Cats were pretreated with 1 mg/Kg HC-3, 5 min prior to the onset of stimulation. The data are expressed as a percentage of the values obtained in the contralateral resting ganglia, and each point given represents the mean ± S.E. of 4 to 6 experiments. $P < 0.05$ as compared to the values obtained in resting unstimulated ganglia.

Fig. 5. Effects of Preganglionic Stimulation at Different Frequencies and HC-3 (1 mg/Kg) on the Isometric Contractile Response of the Nictitating Membrane



The representative experiments illustrating the effects of preganglionic stimulation at different frequencies on the isometric contractile response of the nictitating membrane are depicted. Cats were pretreated with 1 mg/Kg HC-3, 5 min prior to the onset of stimulation.

Fig. 6. Time-Course Effects of HC-3 (1 mg/Kg) in Ganglia Stimulated at 20/Sec on ACh and Choline Content



Time-course effects of preganglionic stimulation at 20/sec on ganglionic content of ACh and choline are depicted. Cats were pretreated with HC-3 5 min prior to the onset of stimulation. The data are expressed as a percentage of the values obtained in the resting unstimulated ganglia and each point represents mean ± S.E. of 4 to 6 experiments. * $p < 0.05$ as compared to the values obtained in resting unstimulated ganglia.

HC-3 on ganglionic ACh stores. However, after ten min of stimulation there was a further gradual decline in the contractile response and after 30 min of stimulation, transmission was almost completely blocked despite the fact that these ganglia still retained about 50% of their original stores of ACh. These data suggest that the membrane response may be a poor index of inhibitory effect of HC-3 on ACh synthesis. Under these experimental conditions HC-3 did not significantly alter choline levels at any time period.

To investigate further the time course effect of HC-3 at 2/sec was studied and compared with that at 20/sec (Fig. 7). As expected at 2/sec the rate of ACh depletion was much slower than 20/sec and significant changes in ACh content were only observed after 20 min of stimulation. However, despite the marked reduction (42%) of ACh stores, after 20 min of stimulation, no failure of transmission was evident. These data further support the observation that the physiological response of the tissue to cholinergic nerve stimulation may be an unreliable index of the ability of HC-3 to inhibit ACh synthesis.

The time-course effects of HC-3 on ACh content of ganglia stimulated at 20/sec reveal that there is a delay of about 5 min in the onset of action of this drug, and that the maximum depletion (50%) is attained after 10 min of stimulation (Fig. 7). Prolonging the stimulation period beyond 10 min does not cause any significant further decrease in ACh stores. The delayed onset of the drug effect may be due to several factors: (1) it may take a few minutes before maximal inhibitory effect of HC-3 can be produced on the choline transport system, and (2) there may be a limited store of choline in nerve terminals (Friesen *et al.*, 1967) which can be utilized for ACh synthesis. It is

Fig. 7. Time-Course Effects of HC-3 (1 mg/Kg) in Ganglia Stimulated at 2 and 20/Sec on Acetylcholine.

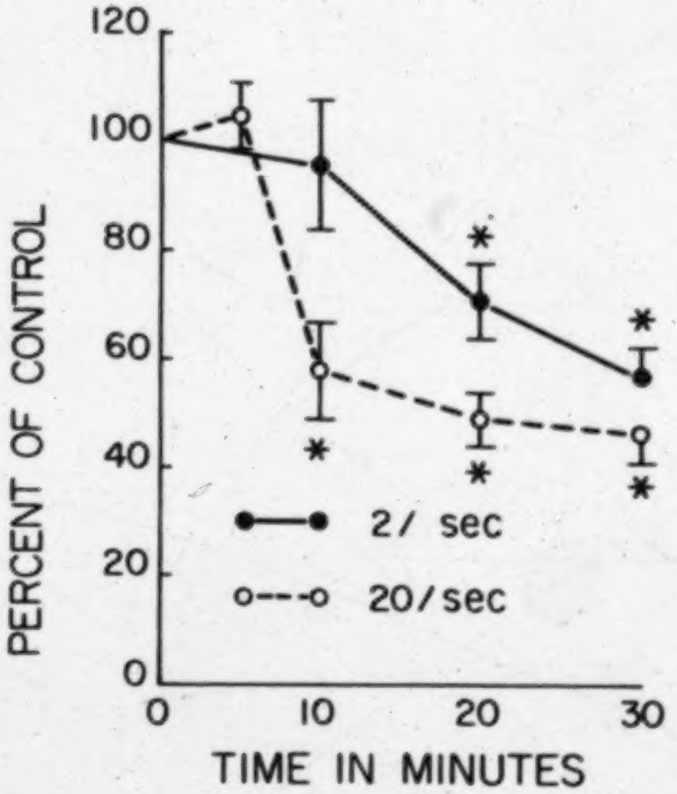


Fig. 7 illustrates the influence of HC-3 on ACh in ganglia stimulated at 2 and 20/sec. The values represent the mean ± S.E. of 4 to 6 experiments. The data are expressed as percentage of the values obtained in the contralateral resting ganglia. $P < 0.05$ as compared to the values obtained in resting unstimulated ganglia.

even more difficult to explain why the degree of ACh depletion did not exceed 50% in any of the experiments involving the use of HC-3. Some possible explanations may include: (1) the available choline may be more efficiently converted to ACh as the concentration of ACh falls in the vicinity of choline acetyltransferase, (2) HC-3 may raise the blood levels of choline by blocking the renal excretion (Sung and Johnstone, 1965) and by inhibiting the uptake of choline into other tissues, thereby limiting its own action on choline transport in ganglia, and (3) HC-3 may, at a later time, inhibit ACh release by a mechanism unrelated to its effects on ACh synthesis. In view of these results it was decided to investigate the time-course effects of a higher dose (2 mg/Kg) of HC-3 on the ACh and choline levels of ganglia. Simultaneous studies were carried out on the effects of this high dose (2 mg/Kg) of HC-3 on the plasma choline levels.

The time-course effects of a 2 mg/Kg dose of HC-3 on ganglionic ACh and choline content are presented in Table IV. For comparative purposes the data obtained at the 1 mg/Kg dose of HC-3 are also included. At a dose of 2 mg/Kg HC-3 the ACh content was reduced by about 50% during the first 5 min of stimulation (Fig. 8), thereafter the rate of decline in the ACh stores was much slower such that after a total of 30 min the content was reduced by 75%. It is noteworthy that at a dose of 2 mg/Kg HC-3 the most rapid decline (50%) in ACh stores occurred within the first 5 min of stimulation. This finding strongly suggests that the readily mobilizable pool of ACh represents 50% of the total stores, and that the remainder is slowly converted to a releasable form. However, the values obtained for the choline content under these conditions do not seem to be consistent with the hypothesis associated with

TABLE IV

Effect of HC-3 on the ACh and Choline content of ganglia stimulated at a frequency of 20/sec.

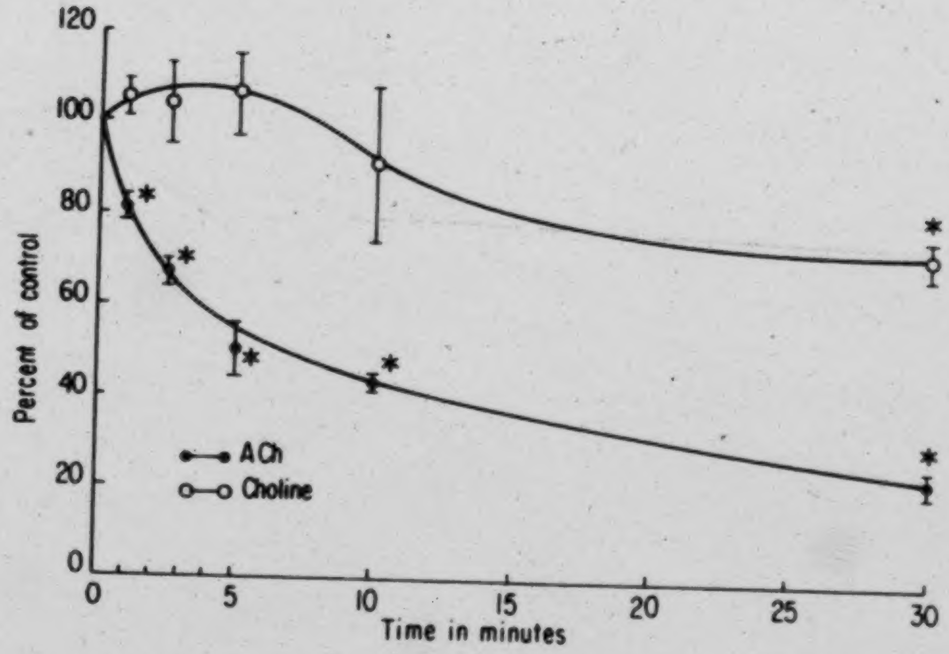
Duration of Stimulation in Minutes	Content as a % of the Resting Contralateral Ganglion			
	ACh		Choline	
	1 mg/Kg HC-3	2 mg/Kg HC-3	1 mg/Kg HC-3	2 mg/Kg HC-3
1	-	81 ± 3*	-	105 ± 4
2.5	-	67 ± 3*	-	104 ± 9
5	105 ± 6	50 ± 6*	101 ± 13	106 ± 9
10	58 ± 9*	43 ± 2*	92 ± 10	91 ± 17
20	50 ± 5*	-	99 ± 15	-
30	46 ± 5*	23 ± 3*	100 ± 7	72 ± 4*

Values given are means ± S.E. of 5 to 8 experiments.

* $p < 0.05$ as compared with values obtained in the corresponding unstimulated contralateral ganglia.

HC-3 was administered 5 min prior to the onset of preganglionic stimulation.

Fig. 8. Time-Course Effects of HC-3 (2 mg/Kg) in Ganglia Stimulated at 20/Sec on ACh and Choline Content



Time-course effects of preganglionic stimulation at 20/sec on ACh and choline content are illustrated in Fig. 8. Cats were pretreated with 2 mg/Kg HC-3, 5 min prior to the onset of stimulation. The data are expressed as a percentage of the values obtained in contralateral unstimulated ganglia. Each point represents mean ± S.E. of 4 - 6 experiments. * $p < 0.05$ as compared to values obtained in unstimulated control ganglia, (0—0) represents ACh, (0--0) represents choline.

the mechanism of action of HC-3.

The depletion of ACh (53 ng) which occurred with 2 mg/Kg HC-3 during the initial 1 min period of stimulation (Table IV), is somewhat greater than the quantity which could have been released at 20/sec in absence of HC-3 [based on the volley output (35 μμg) of ACh from plasma perfused ganglia as determined by Birks and MacIntosh, 1961]. A comparison of their results with present studies on blood perfused ganglia suggests the following hypotheses. Since the initial minute and volley outputs of ganglia exposed to HC-3 are greater than that observed in plasma perfused ganglia, the data indicate that (1) At this dose level HC-3 immediately blocks ACh synthesis completely, and as discussed earlier may also inhibit the utilization of choline within presynaptic nerve terminals. (2) HC-3 may increase the initial volley output of ACh or alternatively, the initial volley output of blood perfused ganglia may be somewhat greater than that which can be achieved in plasma perfused ganglia.

To verify that the 2 mg/Kg dose of HC-3 does, in fact, block completely the ACh synthesis throughout the total 30 min period of stimulation, we investigated the time-course effects of a still higher dose (4 mg/Kg) of HC-3. While it was felt certain that synthesis of ACh was effectively inhibited during the first 10 min, we were less sure about the blockade after 10 min of stimulation in presence of 2 mg/Kg HC-3. In other words does the rate of ACh depletion decrease beyond 10 min because the remaining ACh is slowly mobilized for release, or are significant quantities of ACh now being synthesized? In addition, if HC-3 does enhance transmitter release initially, a higher dose could be expected to cause a further increase in the apparent volley output during the first min of stimulation.

The time-course effect of 4 mg/Kg HC-3 on ACh content of stimulated (20/sec) ganglia are presented in Table V. For comparative purposes the data reported on 1 mg/Kg and 2 mg/Kg doses of this drug (Table IV) are also included. Hemicholinium no. 3 at the level of 4 mg/Kg dose failed to produce a greater depletion of ACh stores than obtained with 2 mg/Kg dose of HC-3. Since at a 4 mg/Kg dose of HC-3 the depletion of ACh is even less than at 2 mg/Kg, the data indicates that the initial volley output of ACh is probably not affected by HC-3. The data also suggest that 2 mg/Kg of HC-3 may be an optimum dose and is capable of immediately blocking ACh synthesis completely provided that drug is given prior to the onset of stimulation.

To determine if HC-3, in absence of stimulation, would cause alterations in ganglionic ACh or choline content, we decided to study the effects of 2 and 4 mg/Kg doses of HC-3 on ACh and choline content of resting (unstimulated) ganglia. The effect of 35 min exposure of resting ganglia to HC-3 are illustrated in Table VI. The data clearly indicate that HC-3 even up to the dose of 4 mg/Kg does not significantly affect either the ACh or choline stores of ganglia under these conditions.

The effects of preganglionic stimulation (20/sec), in presence of HC-3, on choline content of ganglia are summarized in Table VII. Only in one case did HC-3 significantly lower the choline levels; this occurred with 2 mg/Kg HC-3 after 30 min of stimulation. The data clearly indicate that HC-3 is capable of depleting the ACh content of ganglia without reducing the choline levels. The data further suggest that HC-3 may not only block the uptake of choline from the extracellular fluid but may also prevent the utilization of choline within the nerve terminals. Al-

TABLE V

Effect of HC-3 and preganglionic stimulation at a frequency of 20/sec on the ACh content. ACh content expressed as a % of the value obtained in the resting contralateral ganglion.

Minutes of Stimulation	1 mg/Kg HC-3	2 mg/Kg HC-3	4 mg/Kg HC-3
1	-	81 ± 3*	85 ± 4*
2.5	-	67 ± 3*	-
5	105 ± 6	50 ± 6*	58 ± 2*
10	58 ± 9*	43 ± 2*	51 ± 3*
20	50 ± 5*	-	-
30	46 ± 5*	23 ± 3*	40 ± 3*

Values given are means ± S.E. of 5 to 10 experiments.

HC-3 was administered intravenously 5 min prior to the onset of stimulation.

* $p < 0.05$ as compared with values obtained in the corresponding unstimulated contralateral ganglia.

TABLE VI

The ACh and choline content of resting ganglia exposed to HC-3 for 35 min. Content expressed as a % of the values obtained in contralateral ganglia not exposed to HC-3.

Dose of HC-3	ACh	Choline
2 mg/Kg	97 ± 2	95 ± 6
4 mg/Kg	103 ± 4	108 ± 7

Values given in the table are means ± S.E. of 6 to 10 experiments.

TABLE VII

Effect of HC-3 and preganglionic stimulation at a frequency of 20/sec on the choline content. Choline content as a % of the value obtained in the resting contralateral ganglion.

Minutes of Stimulation	1 mg/Kg HC-3	2 mg/Kg HC-3	4 mg/Kg HC-3
1	-	105 ± 4	92 ± 6
2.5	-	104 ± 9	-
5	101 ± 13	106 ± 9	88 ± 9
10	92 ± 10	91 ± 17	87 ± 6
20	99 ± 15	-	-
30	100 ± 7	72 ± 4*	87 ± 8

Values given are means ± S.E. of 5 to 10 experiments.

HC-3 was administered intravenously 5 min prior to the onset of stimulation.

* $p < 0.05$ as compared with the values obtained in the corresponding unstimulated contralateral ganglia.

ternatively choline stored in these nerve terminals (Friesen *et al.*, 1967) is largely unavailable for ACh synthesis.

In order to investigate if HC-3 inhibits its own action by raising the blood levels of choline and thus maintains a continuous supply of choline for ACh synthesis, we studied the effects of different doses of HC-3 on plasma choline levels at different time periods. Since MacIntosh (1963) has reported that major pelvic and abdominal surgery or cortisone administration reduces plasma choline levels, we also determined the plasma choline concentration in absence of HC-3. However, it is evident from the results (Table VIII) that the surgical procedures involved in exposing these ganglia do not constitute a sufficient stress to significantly lower the concentration of choline. It can be seen that while 2 mg/Kg HC-3 did not alter the plasma level of choline, a dose of 4 mg/Kg caused a significant increase of about 50%. However, based on the results of other workers (Birks and MacIntosh, 1961) even this increase of 50% is too small to significantly antagonize the action of HC-3. Thus the results demonstrate that HC-3 can seriously impare ACh synthesis in cholinergic nervous system without appreciably altering the concentration of choline in plasma.

E. Effects of Preganglionic Stimulation and HC-3 on the Ultrastructure of Ganglia and the Relationship of these Effects to the Acetylcholine Content

The major objective of research of this section was to establish what relationships, if any, exist between the ACh content of the superior cervical ganglion of the cat and the number of agranular

TABLE VIII

Effect of HC-3 on the concentration of choline in cat plasma. Plasma choline levels as a % of pre-surgery levels.

Time in Minutes After Surgery	No HC-3	2 mg/Kg HC-3	4 mg/Kg HC-3
0[†]	90 ± 5	90 ± 5	112 ± 14
5	-	93 ± 7	151 ± 23
10	-	-	153 ± 27
15	88 ± 8	99 ± 7	-
30	85 ± 8	100 ± 3	158 ± 19*

Values given are means ± S.E. of 5 to 9 experiments.

The plasma choline concentration prior to surgery was 6.5 nMoles/ml (N = 23).

* $p < 0.05$ as compared with the values obtained prior to surgery.

[†] HC-3 was administered immediately after this blood sample was drawn at the completion of the surgical procedure involved in exposing the superior cervical ganglia.

vesicles. In this regard ganglionic content of ACh was altered by different experimental procedures and the corresponding ultrastructural changes in the nerve terminals with respect to both agranular and dark-core vesicles were investigated. The conditions applied to alter the ACh content of ganglia were the same as described in the first two parts of the result section, e.g., stimulation at different frequencies or treatment with HC-3.

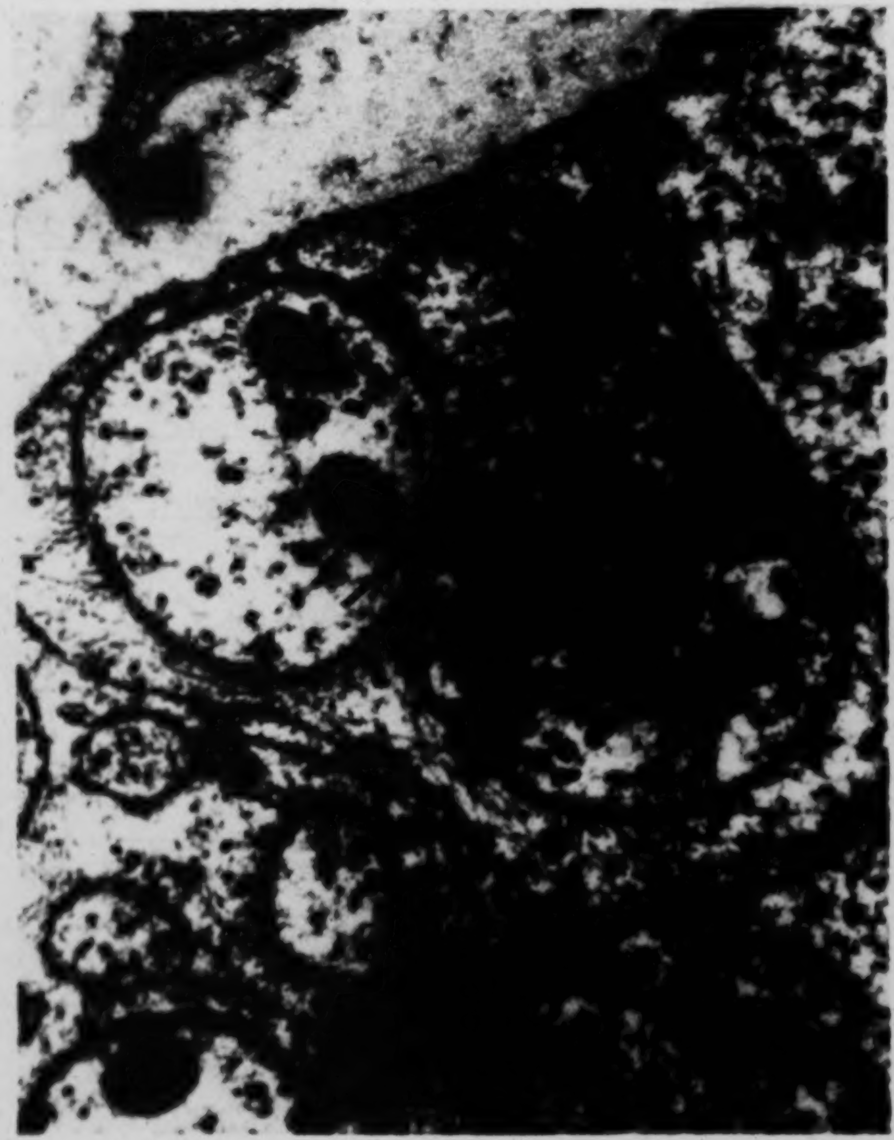
1. Electron micrographs of preganglionic nerve terminals of resting (unstimulated) control ganglia

Electronmicrographs of control unstimulated nerve endings are presented in Fig. 9 and Fig. 10. These pictures reveal presynaptic nerve terminals with their normal content of synaptic vesicles and Fig. 10 also illustrates the typical structure of mitochondria which are frequently found in these nerve endings (L.G. Elfvin, 1963). The dark region separating the pre- and post-synaptic membranes, as shown by the arrow, is recognized as the synaptic cleft.

The population of synaptic vesicles, either agranular or dark-core, in preganglionic nerve terminals is expressed as the number of vesicles per μ^2. To obtain these figures, the total number of respective vesicles in the nerve ending were counted and the total area of the nerve ending as depicted on the electronmicrographs was determined in μ^2. The number of vesicles per μ^2 was then calculated.

The frequency distribution of the number of agranular vesicles per μ^2 in different nerve endings is illustrated in Fig. 11. All nerve endings from resting control ganglia and all other data from experiments in which the number of agranular vesicles did not differ from the con-

Fig. 9. The electronmicrograph represents a preganglionic nerve ending of a resting control ganglion.



NE represents presynaptic unstimulated control nerve ending.

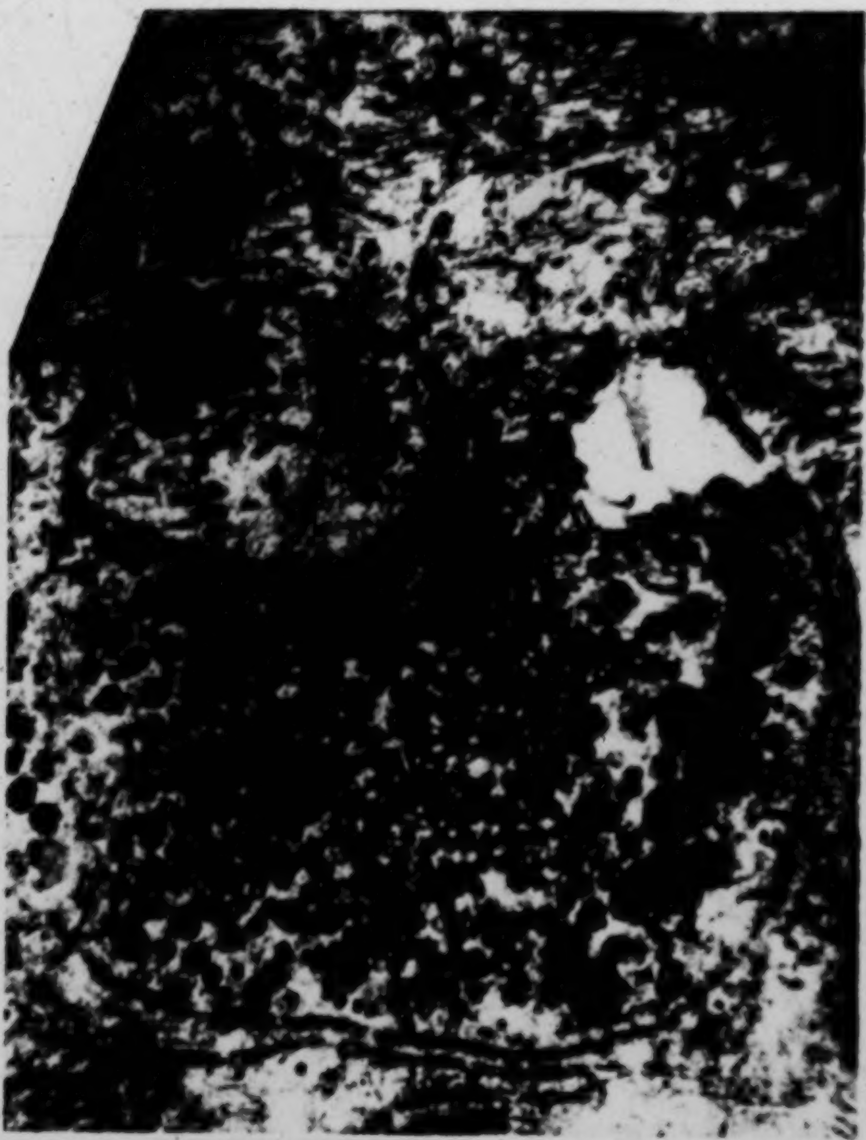
Arrow indicates the synaptic region.

M indicates the presynaptic mitochondria.

AV indicates the agranular vesicles.

DV indicates the dark-core vesicles.

Fig. 10. The electronmicrograph represents a preganglionic nerve ending of a resting control ganglion.



NE represents presynaptic unstimulated control nerve ending.

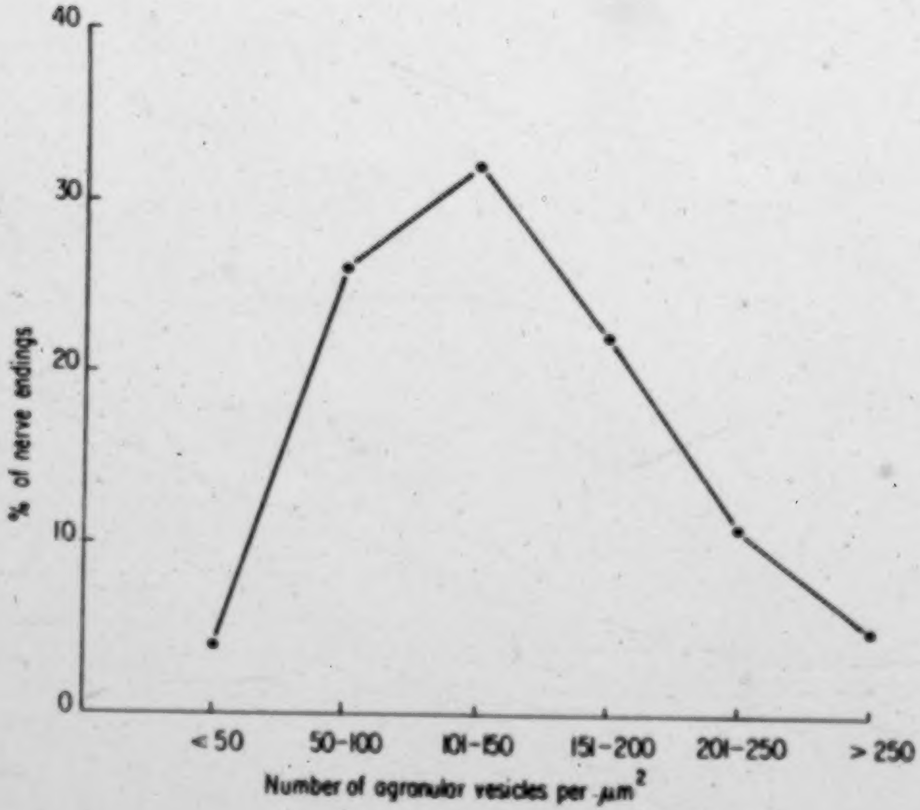
Arrow indicates the synaptic region.

M indicates the presynaptic mitochondria.

AV indicates the agranular vesicles.

DV indicates the dark-core vesicles.

Fig. 11. Frequency distribution of the number of agranular vesicles per μ^2 in different nerve endings.



The variation in number of agranular vesicles in different nerve endings is depicted. A total of 240 nerve endings taken from resting unstimulated ganglia and from all other ganglia in which the number of agranular vesicles did not differ statistically from controls were included in this survey. At least 5 nerve endings were taken from each ganglia and a total of 40 ganglia were used in this analysis.

trol were included in this survey. While the greatest percentage of nerve endings contain 100 to 150 agranular vesicles per μ^2, about 5% had less than 50 and about 15% had more than 200 per μ^2. These data emphasize the variability in the population of agranular vesicles in different nerve endings and the need for sampling large numbers of these to establish a statistically significant change.

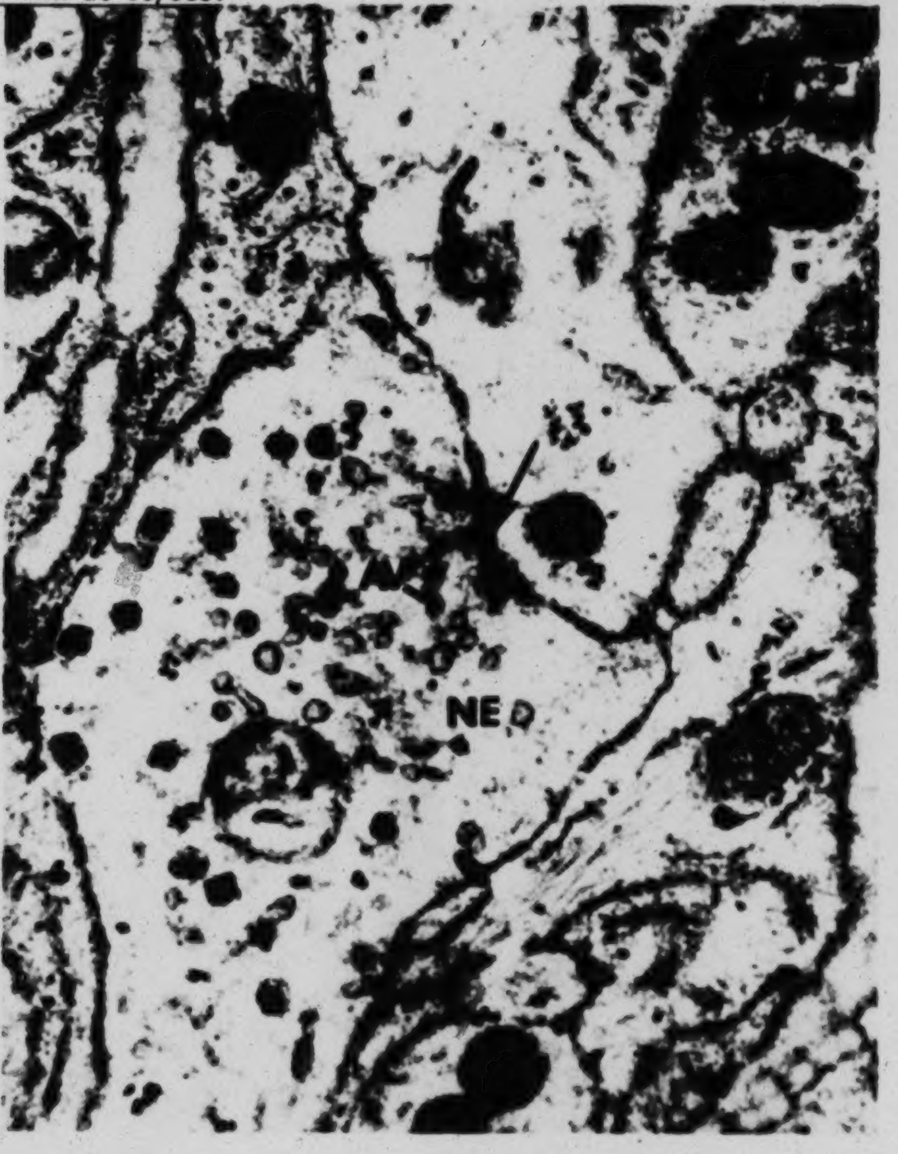
2. Effects of preganglionic stimulation at 60/sec on the number of agranular vesicles in relation to the ACh content of ganglia

a. Stimulation at 60/sec and depletion of agranular vesicles.

We have demonstrated that preganglionic stimulation for 4 min at 60/sec causes a 30% reduction in ACh content (Fig. 1). When the same conditions were applied to investigate the ultrastructure of the nerve endings, marked depletion of synaptic agranular vesicles were observed and many of the remaining vesicles had lost their characteristic conformation. These ultrastructural changes are illustrated in Fig. 12 and Fig. 13. In addition, these stimulation parameters frequently caused the swelling and disruption of mitochondria in presynaptic nerve endings (compare Fig. 10 and 13). However, there was no evidence of alterations in mitochondria in postsynaptic structures.

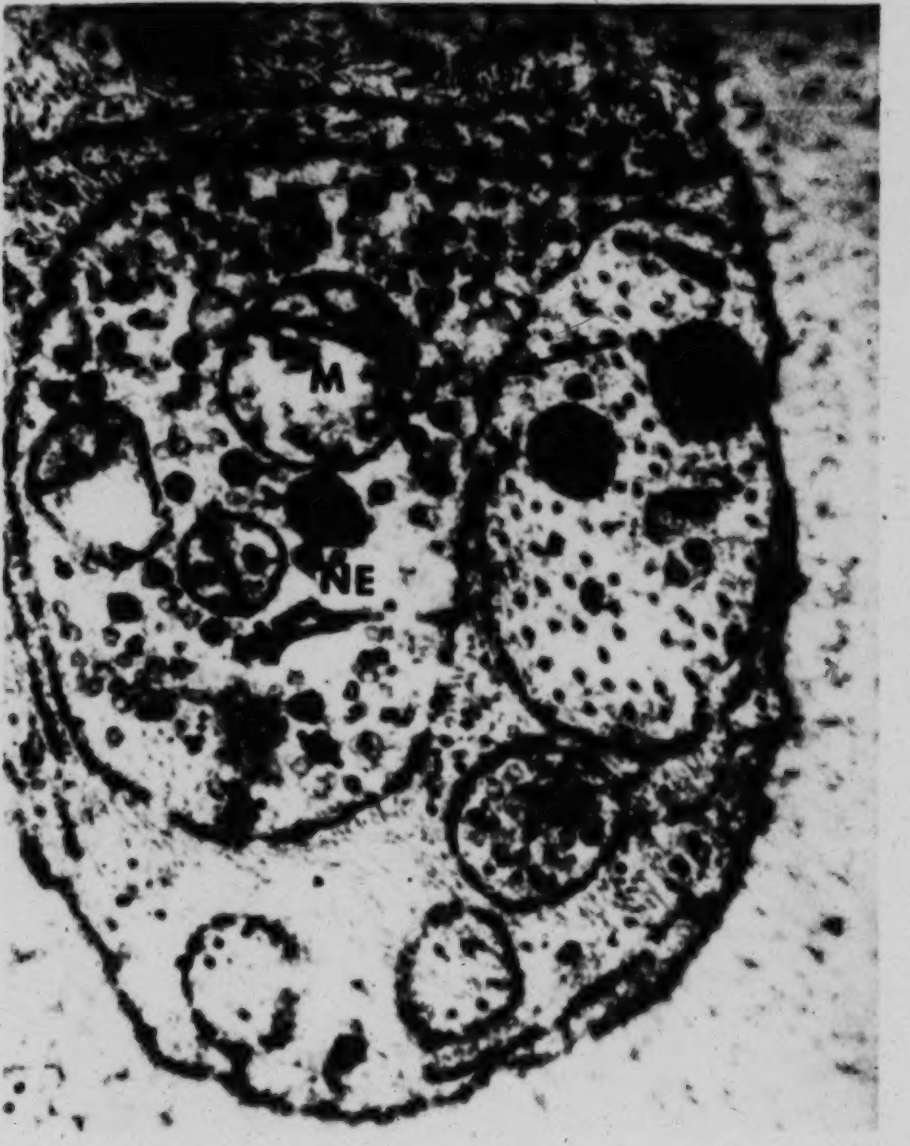
The number of agranular vesicles per μ^2 found under different conditions of stimulation, at 60/sec in total and different regions of nerve endings, are presented in Table IX. When ganglia were stimulated at 60/sec for 4 min to induce a 30% depletion in ACh, a corresponding reduction of about 47% in number of agranular vesicles (Fig. 14) was also observed during this period of stimulation. The results obtained

Fig. 12. The electronmicrograph shows the ultrastructural change in agranular vesicles in preganglionic nerve terminal, when stimulated for 4 min at 60/sec.



NE indicates a preganglionic nerve ending stimulated at 4 min at 60/sec.
Arrow indicates the synaptic region.
AV indicates the agranular vesicles.

Fig. 13. The effects of 4 min of preganglionic stimulation at 60/sec on the ultrastructure of presynaptic mitochondria are depicted.



NE indicates a preganglionic nerve ending stimulated for 4 min at 60/sec.

Arrow indicates the synaptic region.

M indicates the presynaptic mitochondria.

TABLE IX

Effects of preganglionic stimulation at 60/sec on the agranular vesicles of total, synaptic and non-synaptic regions of nerve endings.

	Number of Agranular Vesicles per μ^2		
Experimental Condition	Total	Synaptic	Non-synaptic
a. Resting Controls	141 ± 6.5	190 ± 11.5	134 ± 7.4
b. Stimulation for 4 min	75 ± 4.6*	153 ± 11.5	62 ± 5.1*
c. Stimulation for 4 min + 2 min rest	94 ± 6.2*	205 ± 17.6	75 ± 6.2*
d. Stimulation for 4 min + 20 min rest.	125 ± 12.3[+]	212 ± 18.1	112 ± 11.6[+]

---

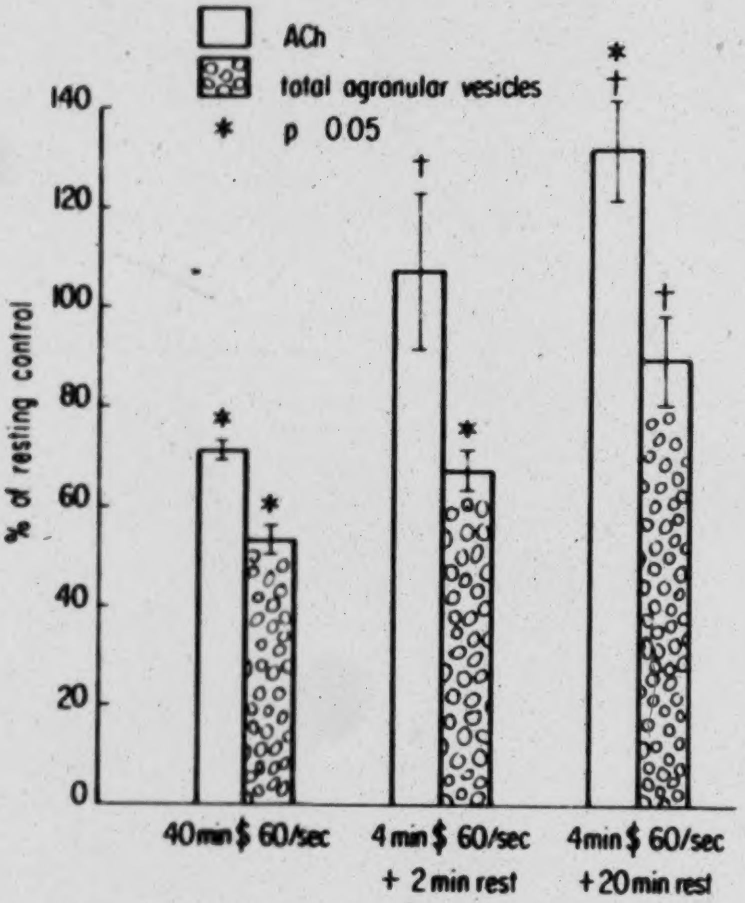
[1] Within the zone of 2000 Å from the synaptic cleft.

The values are mean ± S.E. of 40 nerve endings taken from 5 to 7 ganglia.

* $p < 0.05$ as compared to the values obtained in resting control ganglia.

[+] $p < 0.05$ as compared to the values obtained from ganglia stimulated for 4 min at 60/sec.

Fig. 14. Effects of preganglionic stimulation at 60/sec on the number of agranular vesicles and ganglionic acetylcholine content.



A correlation between the number of agranular vesicles and the ganglionic ACh content in stimulated (60/sec) ganglia is depicted. The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case. NS represents the number of agranular vesicles in non-synaptic area. S indicates the number of agranular vesicles in synaptic zone. * p<0.05 as compared to the values obtained in unstimulated resting ganglia. † p<0.05 as compared to the values obtained in ganglia stimulated 4 min at 60/sec.

are consistent with the concept that agranular vesicles are the storage sites of ACh.

Since agranular vesicles are generally found more concentrated (Table IX) near the presynaptic membrane, we decided to investigate whether the distribution of the vesicles near the synaptic membrane or the vesicles farther away from the synaptic membrane were affected by the above stimulation parameters. Results illustrated in Fig. 15 represent the ganglionic content of ACh and the distribution of agranular vesicles in the synaptic zone of 2000 Å (as defined in methodology section) and in the non-synaptic area under different conditions of stimulation at 60/sec. It can be seen that significant depletion in agranular vesicles occurred mainly in the non-synaptic area. Since there appears to be less depletion (19%) in the synaptic area, this may indicate that the vesicles from the non-synaptic zone replace those depleted from the synaptic region during stimulation.

b. Recovery in number of agranular vesicles in relation to the recovery of ganglionic ACh content during the rest period after initial preganglionic stimulation at 60/sec.

We have already demonstrated (Fig. 2) that ganglia recover their lost ACh content within 2 min of rest period after 4 min of initial preganglionic stimulation at 60/sec. It was, therefore, felt important to ascertain if a corresponding recovery in agranular vesicles also occurs during this resting period.

When allowed to rest after 4 min of initial stimulation at 60/sec, although recovery in ACh content occurred within 2 min of rest, no statistically significant recovery in number of agranular vesicles was observed (Fig. 14). However, when allowed to rest for 20 min to induce a 30% rebound increase (Fig. 2) in ACh content, although a significant recovery in number of vesicles occurred (Fig. 14) their density did not rise above the control values. This latter situation represents a case where changes in ACh content are not reflected in corresponding changes in the number of agranular vesicles.

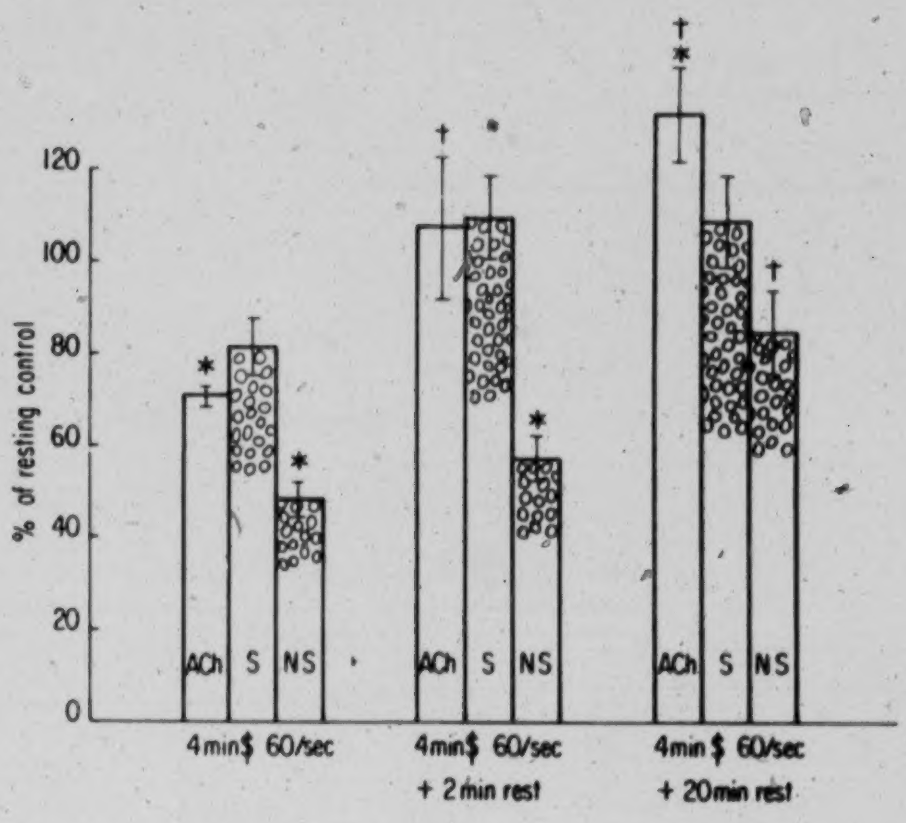
The effects of the rest periods on the distribution of agranular vesicles, in the synaptic and non-synaptic area are illustrated in Fig. 15. It can be seen that after 2 min of rest although there was some apparent recovery in the number of vesicles in the synaptic area, the increase in the number of vesicles was not statistically different from the number of vesicles observed after 4 min of stimulation at 60/sec. After 20 min of rest, significant recovery in the number of vesicles occurred in the non-synaptic area, but their density did not exceed control values.

3. Effects of hemicholinium no. 3 (2 mg/Kg) and preganglionic stimulation at a frequency of 20/sec on the number of agranular vesicles

In this series of experiments HC-3 (2 mg/Kg) was used to induce alterations in ganglionic ACh content and the corresponding ultrastructural changes in the nerve terminals were investigated.

As presented in Fig. 8, ganglia lose about 50% of their ACh store within 5 min of stimulation in presence of HC-3. The results concerning the changes in number of agranular vesicles under the same conditions of stimulation are illustrated in Fig. 16. When ganglia were stiulated for 5 min in absence of HC-3, there was no significant change in

Fig. 15. Influence of preganglionic stimulation for 4 min at 60/sec on the density of agranular vesicles in different regions of nerve endings.

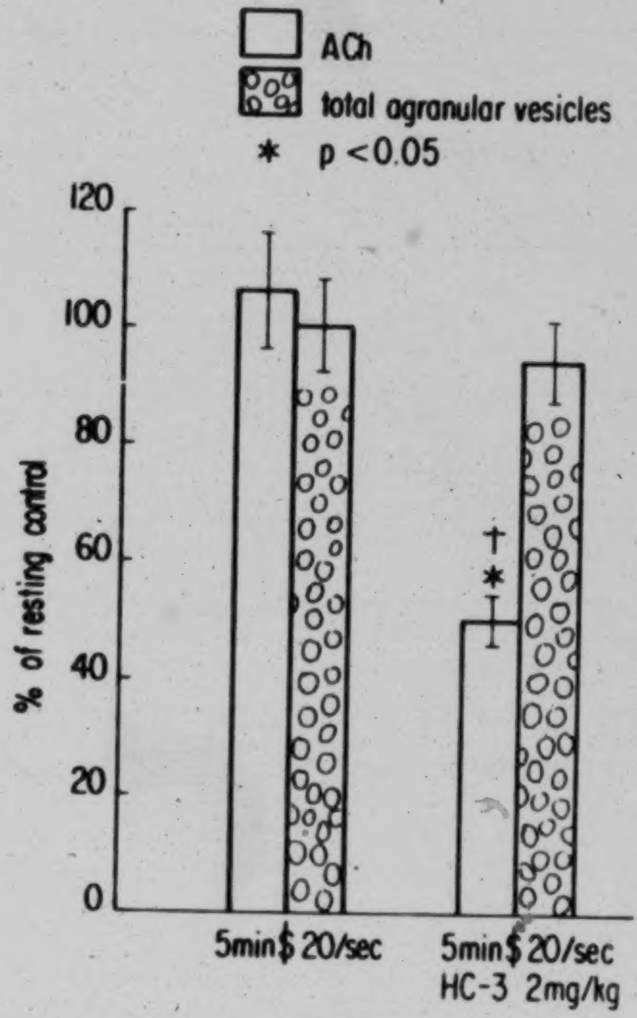


The bar graphs illustrate the effects of stimulation at 60/sec on the population of vesicles in synaptic zone (S) and non-synaptic area (NS) of nerve endings. The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case.

* $p < 0.05$ as compared to the values obtained in unstimulated resting ganglia.

† $p < 0.05$ as compared to those obtained in ganglia stimulated for 4 min at 60/sec.

Fig. 16. Effects of HC-3 (2 mg/Kg) and preganglionic stimulation at 20/sec, on the population of agranular vesicles in the nerve endings, and on ganglionic ACh content.



The bar graphs illustrate the inability of stimulation at 20/sec for 5 min to deplete the vesicles in ganglia either untreated or pretreated with HC-3. The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case.

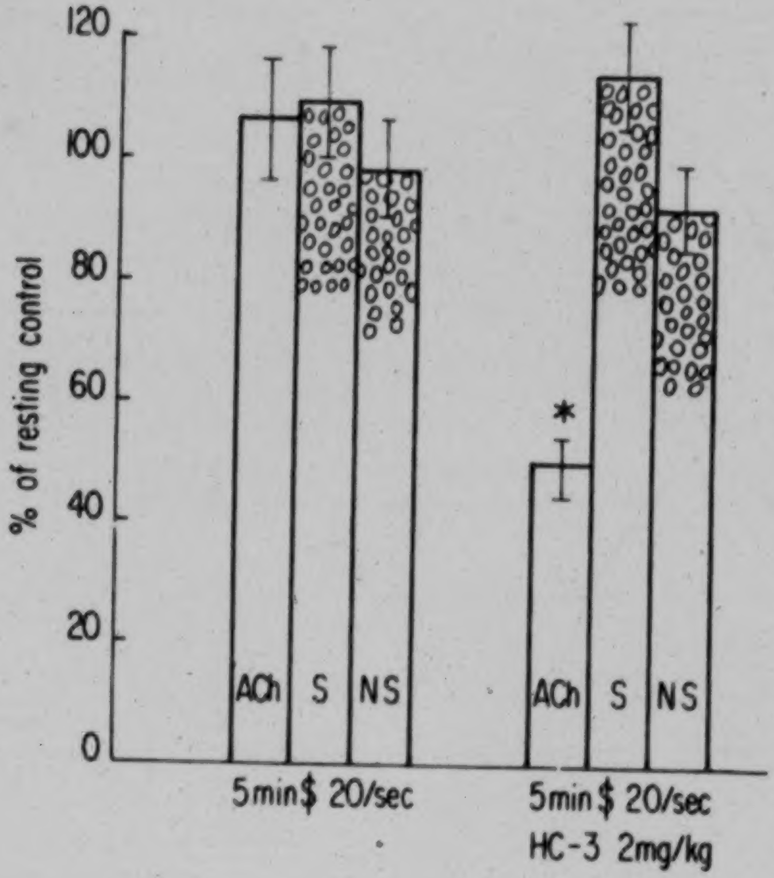
* p < 0.05 as compared to the values obtained in unstimulated resting ganglia.

either ACh content or the number of agranular vesicles. On pre-treatment with HC-3 (2 mg/Kg), although ganglia lost about 50% of ACh within 5 min of stimulation (Fig. 16), no corresponding decrease in the number of agranular vesicles was found. The distribution of vesicles in the synaptic zone and non-synaptic area (Fig. 17) were also unaffected by these conditions of stimulation. This represents another example in which there was no correspondence between ACh content and the number of agranular vesicles in the nerve terminals.

Similarly no direct correlation between ACh content and the number of vesicles was observed when ganglia were stimulated for 30 min in absence of HC-3. In this case preganglionic stimulation at 20/sec for 30 min (in the absence of HC-3) caused a small increase (16%) in the ACh content of ganglia but produced a significant reduction (24%) in number of agranular vesicles (Fig. 18). When ganglia were pretreated with HC-3 and stimulated for the same period a corresponding depletion in both ACh and agranular vesicles occurred (Fig. 19a & b). A 30 min period of stimulation at 20/sec in the presence of HC-3 caused about 75% reduction in the ACh content (Fig. 18) and a 50% decline in population of agranular vesicles. This latter situation is consistent with the concept of vesicles being the storage sites of ACh. Exposure of resting (unstimulated) ganglia to HC-3 for 35 min did not produce any significant alteration in either ACh content of ganglia or in the number of agranular vesicles. The data concerning effects of HC-3 on the number of agranular vesicles are summarized in Table X.

Effects of HC-3 on the ganglionic ACh content and the distribution of agranular vesicles in synaptic and non-synaptic areas are presented in Fig. 20. It can be seen that after 30 min of stimulation,

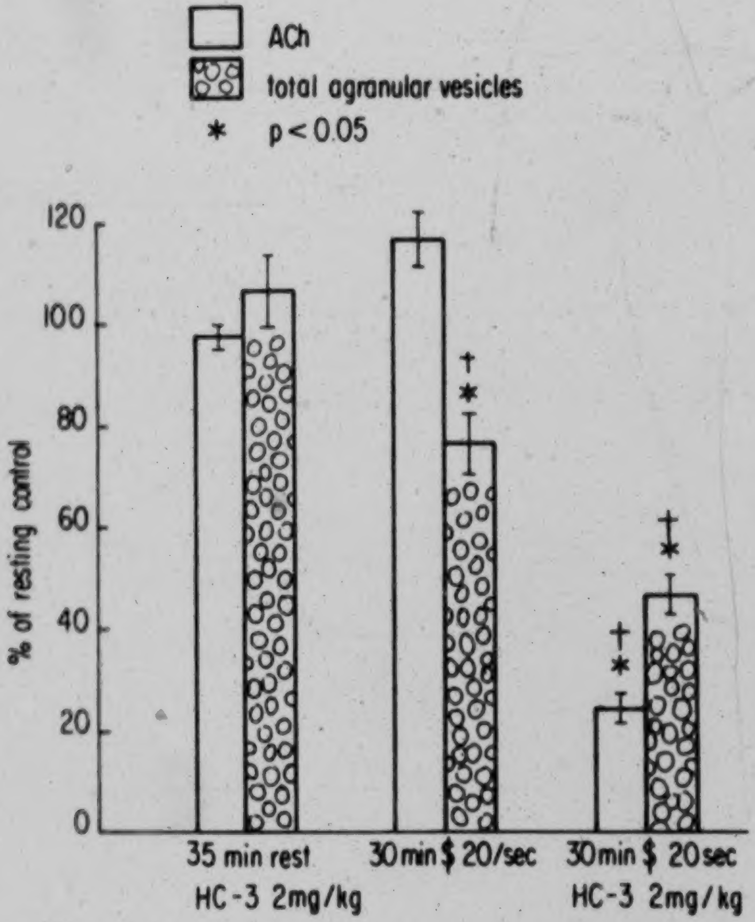
Fig. 17. Influence of HC-3 (2 mg/Kg) and preganglionic stimulation at 20/sec on synaptic and non-synaptic vesicles.



The effects of 5 min stimulation at 20/sec on ganglionic ACh content and the distribution of agranular vesicles in nerve endings, untreated and pretreated with HC-3, are depicted. The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case. NS represents the number of agranular vesicles in non-synaptic area. S represents the number of agranular vesicles in synaptic zone.

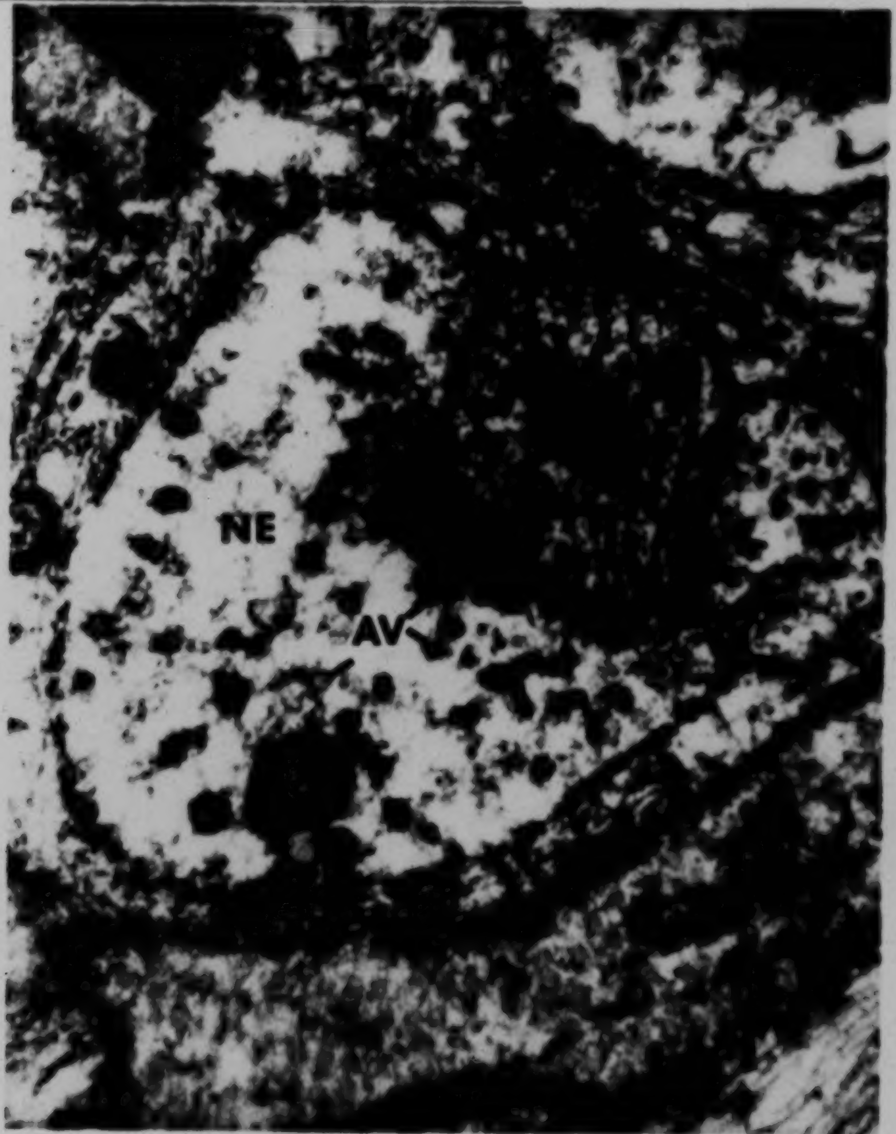
* $p < 0.05$ as compared to the values obtained in unstimulated resting ganglia.

Fig. 18. Effects of HC-3 (2 mg/Kg) and 30 min stimulation at 20/sec on the density of agranular vesicles in preganglionic nerve endings.



The bar graphs illustrate the depletion of agranular vesicles and ganglionic ACh content in ganglia stimulated at 20/sec, in absence or presence of HC-3. In both cases the ganglia were stimulated for a total of 30 min. The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case. * $p < 0.05$ as compared to the values obtained in unstimulated resting ganglia. † $p < 0.05$ as compared to those obtained in ganglia resting for 35 min, pretreated with HC-3.

Fig. 19a. The electronmicrograph represents ultrastructural changes in agranular vesicles in preganglionic nerve terminal, pretreated with HC-3 and stimulated at 20/sec for 30 min.

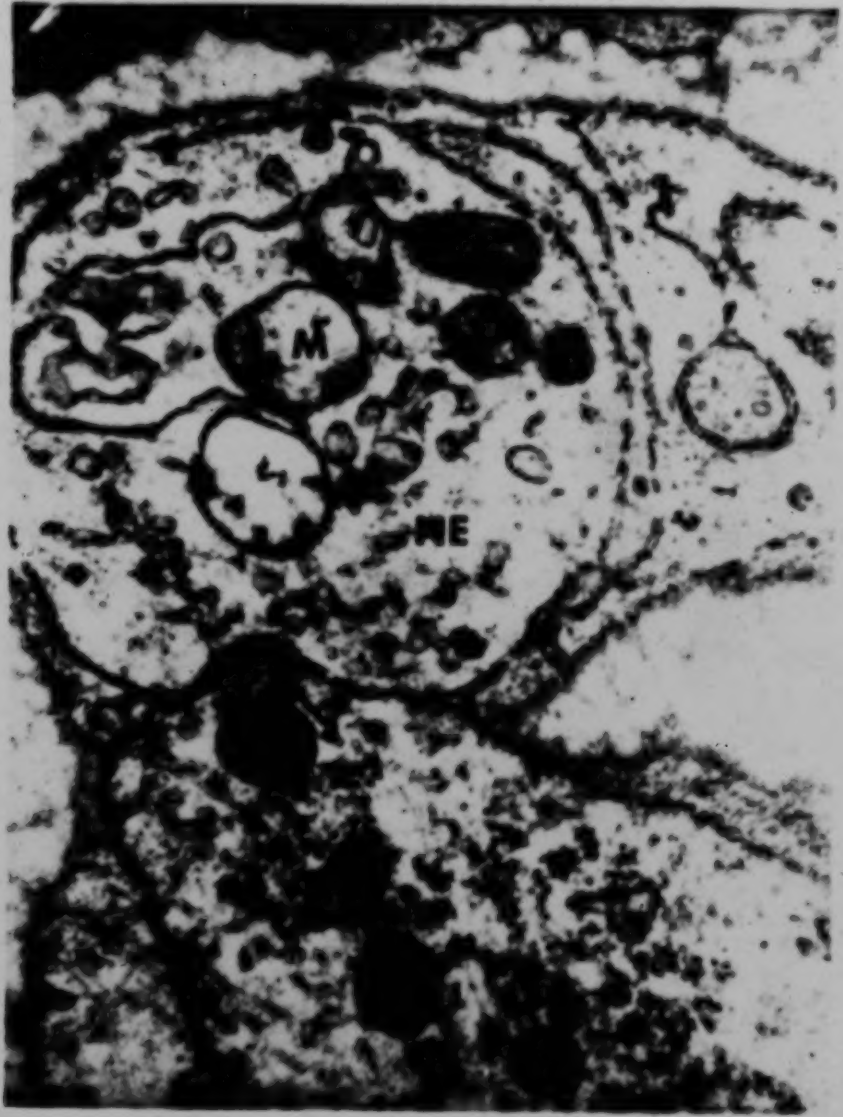


NE represents a preganglionic nerve ending.

Arrow indicates the synaptic region.

AV represents the agranular vesicles.

Fig. 19b. The electronmicrograph represents the effects of HC-3 and preganglionic stimulation at 20/sec for 30 min, on the ultrastructure of presynaptic mitochondria.



NE represents a preganglionic nerve ending.

Arrow indicates the synaptic region.

M represents the presynaptic mitochondria.

TABLE X

Effect of Hemicholinium (2 mg/Kg) and Preganglionic Stimulation at 20/sec on the Agranular Vesicles of Total, Synaptic and Non-synaptic Regions of Nerve Ending.

	Number of Agranular Vesicles per μ^{1}		
Experimental Condition	Total	Synaptic	Non-synaptic
a. Resting controls	141 ± 6.5	190 ± 11.5	132 ± 7.4
b. 5 min stimulation	141 ± 10.6	208 ± 17.7	132 ± 10.4
c. HC-3 and 5 min rest	136 ± 8.9	153 ± 11.2	137 ± 11.7
d. 5 min stimulation and HC-3	133 ± 9.2	219 ± 17.0	123 ± 9.2
e. 30 min stimulation	107 ± 7.8*	190 ± 13.0	99 ± 7.6*
f. HC-3 and 35 min rest	150 ± 9.8	242 ± 12.1	132 ± 9.5
g. 30 min stimulation and HC-3	65 ± 5.9*†	138 ± 12.9	55 ± 5.5*†

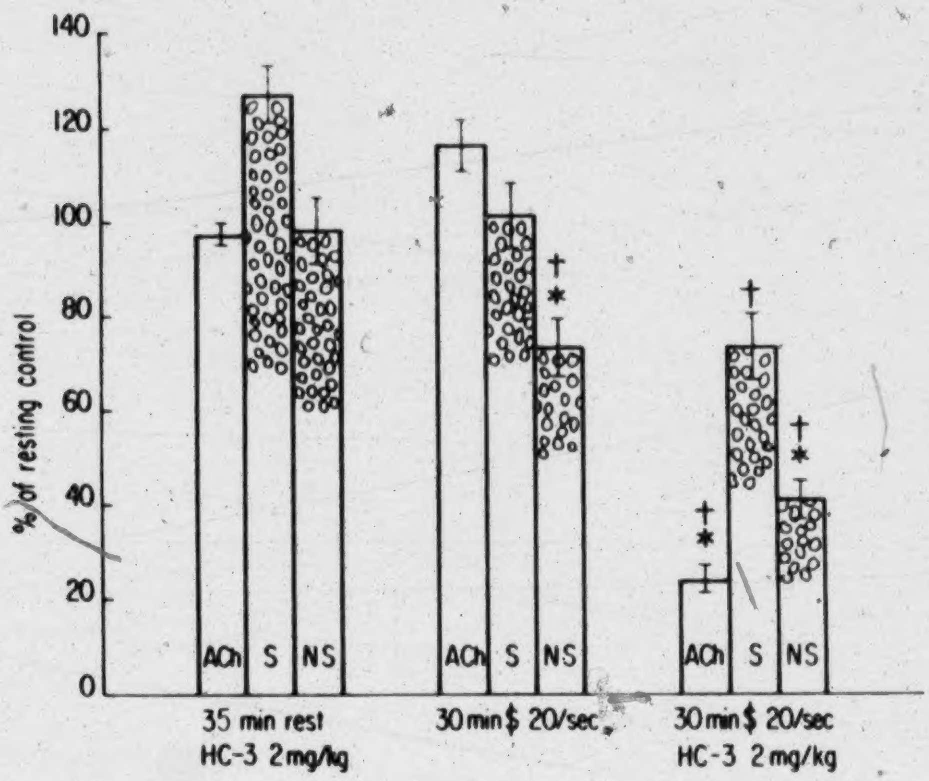
[1] Within a zone of 2000 Å from the synaptic cleft.

The values are mean ± S.E. of 40 nerve endings taken from 5 to 7 ganglia.

* $p < 0.05$ as compared to the values obtained in untreated resting control ganglia.

† $p < 0.05$ as compared to the values obtained in resting ganglia exposed to HC-3 for 35 min.

Fig. 20. Influence of hemicholinium no. 3 and 30 min preganglionic stimulation at 20/sec on the distribution of agranular vesicles in preganglionic nerve endings.



The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case.

NS represents agranular vesicles in non-synaptic zone.

S represents agranular vesicles in synaptic zone.

* $p < 0.05$ as compared to the values obtained in resting unstimulated ganglia.

† $p < 0.05$ as compared to the values obtained in resting ganglia exposed to HC-3 for 35 min.

in absence of HC-3, significant depletion in number of vesicles occurred only in the non-synaptic area. When ganglia were pre-treated with HC-3 this stimulation period caused less depletion of vesicles in synaptic zone than those in non-synaptic area. These data again indicate that the vesicles from the non-synaptic zone may physically move to replace those lost from the synaptic region during stimulation.

To investigate if the alterations in number of agranular vesicles was a true representation and not an artifact due to change in the area of nerve endings, we measured the total and regional nerve ending areas under the above experimental conditions. The results are presented in Table XI. It can be seen that no significant change in the areas was found either in total or any of the regions measured. The data indicate that the alterations in the number of agranular vesicles, caused by the applied experimental condition, were absolute changes and not an artifact due to the change in areas of the nerve endings.

F. Ultrastructural Changes in Dark-Core Vesicles in Relation to the Alterations in Ganglionic Content of Acetylcholine

1. Dark-core vesicles in the nerve terminals of resting (unstimulated)ganglia

A few granular vesicles, 700 - 800 Å in diameter, are often seen in the preganglionic nerve terminals. These granular vesicles constitute about 4% of the total vesicle population and are referred to as dense or dark-core vesicles. Nerve endings of a resting control ganglion are presented in Fig. 9 & 10. It can be seen that dark-core vesicles are mainly localized in the non-synaptic area farther away from the synaptic

TABLE XI

Effects of preganglionic stimulation and hemicholinium no. 3 on the area of nerve endings.

Experimental Condition	Total Nerve Ending Area μ^2	Synaptic Regional Area μ^2	Non-Synaptic Regional Area μ^2
a. Resting controls	1.49 ± 0.12	0.13 ± 0.009	1.36 ± 0.11
b. Stimulation for 4 min (60/sec)	1.37 ± 0.13	0.14 ± 0.011	1.23 ± 0.12
c. Stimulation for 4 min (60/sec) + 2 min rest	1.43 ± 0.13	0.12 ± 0.009	1.31 ± 0.12
d. Stimulation for 4 min (60/sec) + 20 min rest	1.39 ± 0.12	0.12 ± 0.008	1.27 ± 0.12
e. 5 min stimulation (20/sec)	1.33 ± 0.11	0.12 ± 0.008	1.21 ± 0.10
f. 5 min stimulation (20/sec) and HC-3	1.46 ± 0.14	0.13 ± 0.010	1.33 ± 0.14
g. 30 min stimulation (20/sec)	1.61 ± 0.12	0.12 ± 0.007	1.49 ± 0.12
h. HC-3 and rest for 35 min	1.24 ± 0.11	0.13 ± 0.011	1.11 ± 0.11
i. 30 min stimulation (20/sec) and HC-3	1.77 ± 0.15	0.12 ± 0.009	1.65 ± 0.15

The values represent mean ± S.E. of 40 nerve endings in each case.

$p < 0.05$ was used as a level of significance for statistical analysis.

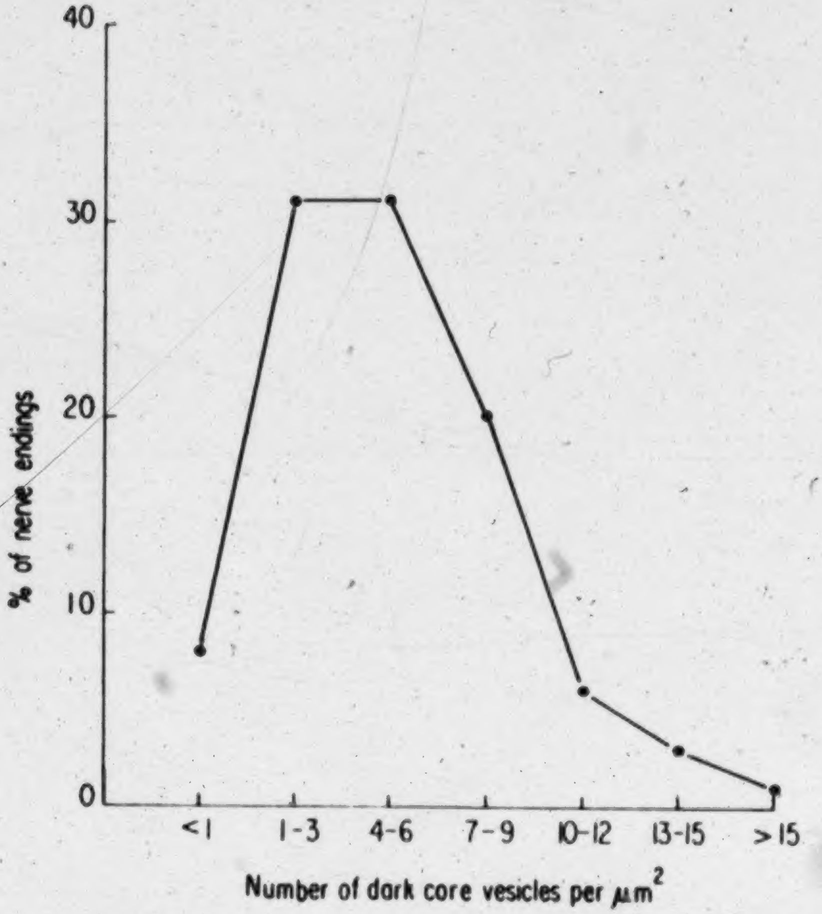
cleft. Although the content of dark-core vesicles is still debatable, it was of interest to evaluate what influence the parameters, which alter ganglionic ACh content, had on the density of these vesicles. As in case of agranular vesicles, the population of dark-core vesicles in preganglionic nerve terminals is expressed as the number of vesicles per μ^2. The number of these dark-core vesicles in the synaptic zone of 2000 Å and non-synaptic area were counted and based on these areas the number of vesicles per μ^2 was calculated.

2. Frequency distribution of the number of dark-core vesicles per μ^2 in different nerve endings

The frequency distribution of these vesicles per μ^2 in different nerve endings is presented in Fig. 21. As in the case of agranular vesicles, all nerve endings from resting control ganglia and all other data from experiments in which the number of dark-core vesicles did not differ from the control were included in this survey. It can be seen (Fig. 21) that there was a considerable variation in their numbers in different nerve terminals. Most nerve endings contain 3 to 6 dark-core vesicles per μ^2, but about 8% contain less than 1 and about 5% contain more than 12 dark-core vesicles per μ^2.

The relationship between the density of dark-core vesicles and the number of agranular is illustrated in Table XII. The number of dark-core vesicles in different nerve endings appeared to be unrelated to the number of agranular vesicles per μ^2. Even in nerve endings which contain more than 12 dark-core vesicles per μ^2, the number of agranular vesicles are not statistically different from those which have a lesser number of dark-core vesicles. The data also indicate that the nerve

Fig. 21. Frequency distribution of dark-core vesicles per μ^2 in different nerve endings.



The variation in number of dark-core vesicles in different nerve endings is depicted. A total of 240 nerve endings taken from resting unstimulated ganglia and from all other ganglia in which the number of dark-core vesicles did not differ statistically from controls, were included in this survey. At least 5 nerve endings were taken from each ganglia and a total of 40 ganglia were used in this analysis.

TABLE XII

Relationship between the number of dark-core and agranular vesicles in the same nerve endings.[2]

	Number of vesicles per μ^2 in nerve endings	
Number of nerve endings	Dark-core	Agranular[1]
17	<1	140 ± 17
66	1 - 3	138 ± 9
82	4 - 6	140 ± 6
45	7 - 9	139 ± 9
16	10 - 12	134 ± 15
12	>12	121 ± 19

---

[1] The values are means ± S.E.

[2] Nerve endings included in this survey were from resting control ganglia and data obtained from all other experimental conditions in which the number of vesicles were not statistically different from controls. A total of 240 nerve endings, taken from 40 ganglia, were used in this analysis. At least 5 nerve endings were taken from each ganglia.

endings used for analysis are probably all of the same type, i.e., preganglionic cholinergic nerve endings.

3. Effects of preganglionic stimulation at 60/sec on the density of dark-core vesicles in the preganglionic nerve terminals

The number and the distribution of dark-core vesicles in 40 control and stimulated (60/sec) nerve endings is presented in Table XIII. It can be seen that most of the dark-core vesicles in resting nerve endings are localized in non-synaptic area. Also there was a large variation in their number in different nerve endings. This is reflected in the standard errors which are very high and vary from 10% in the non-synaptic to 30% in the synaptic zone. Because of these high standard errors, particularly in the data from the synaptic zone, observed changes of 40% to 50% were not statistically significant. On stimulation at 60/sec a significant reduction (37%) in dark-core vesicles corresponding to 30% depletion of ACh content was seen. This marked change in dark-core vesicles occurred mainly in the non-synaptic zone, the data indicates that dark-core vesicles may be mobilized from the non-synaptic to the synaptic zone during stimulation.

During 2 min of rest after initial 4 min of stimulation (60/sec), the number of dark-core vesicles were still comparable to those observed after 4 min stimulation at 60/sec (Fig. 22). When the resting period was extended to 20 min a marked recovery in the density of dark-core vesicles was seen, although their number did not rise above the control values (Fig. 22). These situations are similar to those observed in the case of agranular vesicles and suggest that dark-core vesicles are reused or synthesized during synaptic transmission. The data represent-

TABLE XIII

Number and distribution of dark-core vesicles in preganglionic nerve endings during resting conditions and after stimulation at 60/sec.

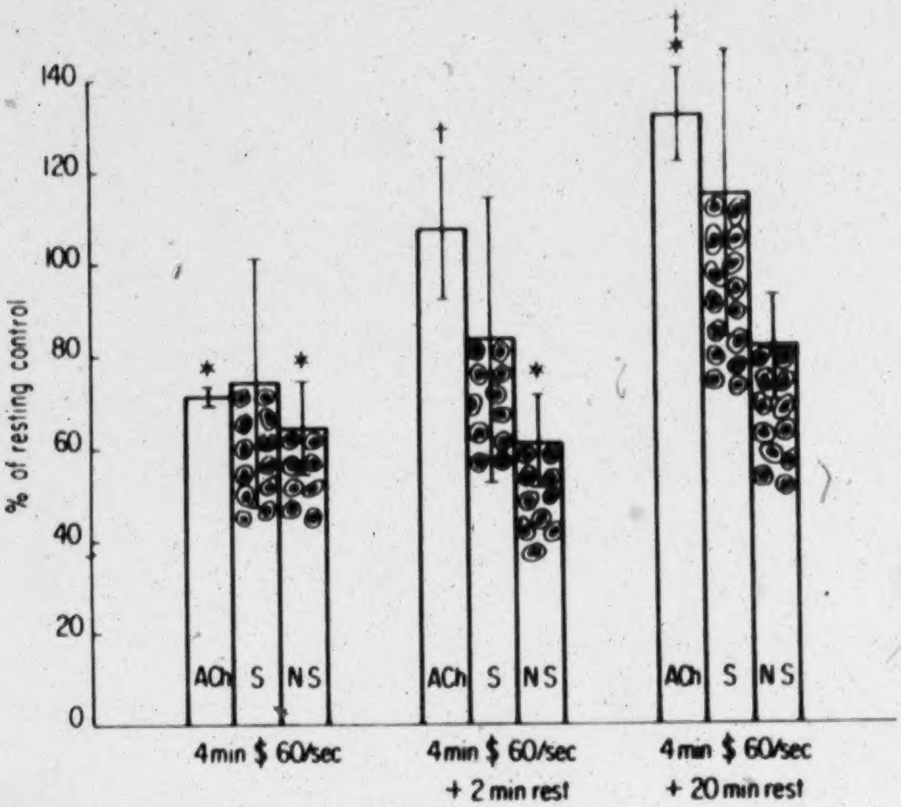
Experimental Condition	Number of Dark-Core Vesicles per μ^2 Total	Synaptic[1]	Non-synaptic
a. Resting controls	5.5 ± 0.54	2.65 ± 0.77	5.90 ± 0.6
b. 4 min stimulation	3.5 ± 0.50*	1.95 ± 0.72	3.75 ± 0.59*
c. 4 min stimulation + 2 min rest	3.3 ± 0.53*	2.20 ± 0.83	3.50 ± 0.56*
d. 4 min stimulation + 20 min rest	4.65 ± 0.61	3.03 ± 0.83	4.80 ± 0.63

[1] Within a zone of 2000 Å from the synaptic cleft.

The values represent mean ± S.E. of 40 nerve endings taken from 5 to 7 ganglia in each case.

* $p < 0.05$ as compared to the values obtained in resting control ganglia.

Fig. 22. Influence of preganglionic stimulation at 60/sec on the number of dark-core vesicles in different regions of nerve endings.



The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case.

S represents the number of dark-core vesicles in synaptic zone.

NS represents the number of dark-core vesicles in non-synaptic area.

* $p < 0.05$ as compared to the values obtained in unstimulated resting ganglia.

† $p < 0.05$ as compared to the values obtained in ganglia stimulated for 4 min at 60/sec.

ing dark-core vesicles in relation to agranular vesicles are presented in Table XIV. It can be seen that the experimental conditions which alter the number of agranular vesicles also have similar effects on dark-core vesicles.

4. Influence of preganglionic stimulation at 20/sec, in absence or presence of HC-3 (2 mg/Kg), on the population of dark-core vesicles

The ultrastructural changes in dark-core vesicles were also evaluated in the nerve terminals stimulated at 20/sec both in absence or presence of HC-3 (Table XV). When ganglia were stimulated for 5 min at 20/sec, untreated or pretreated with HC-3, no significant change in dark-core vesicles (Fig. 23) was seen in any of the areas measured. It is noteworthy that after 5 min stimulation, ganglia pretreated with HC-3 had lost about 50% of the ACh content.

Stimulation for 30 min, in the absence of presence of HC-3, produced a significant depletion in dark-core vesicles (Fig. 24). In the latter situation, this corresponded to a depletion in ACh content. It is noteworthy that statistically significant depletion occurred only in non-synaptic region. Although there was also a 59% depletion in the synaptic dark-core vesicles (Fig. 24), these changes were not statistically significant. The data suggest that similar to agranular vesicles the dark-core vesicles physically move from non-synaptic region to replace those lost during preganglionic stimulation.

The data comparing changes in dark-core and agranular vesicles, when subjected to stimulation and HC-3, are presented in Table XVI. The results demonstrate that both stimulation and HC-3 produce similar effects on the density of agranular and dark-core vesicles in the preganglionic nerve terminals.

TABLE XIV

Effect of preganglionic stimulation at 60/sec on the density of agranular and dark-core vesicles in the preganglionic nerve endings of superior cervical sympathetic ganglia of the cat.

Experimental Conditions	Number of Vesicles per μ^2 of Nerve Ending Agranular	Dark-Core
a. Resting controls	141 ± 6.5	5.5 ± 0.54
b. Stimulation for 4 min	75 ± 4.6*	3.5 ± 0.50*
c. Stimulation for 4 min + 2 min rest	94 ± 6.2*	3.3 ± 0.53*
d. Stimulation for 4 min + 20 min rest	125 ± 12.3†	4.65 ± 0.61

The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case.

* $p < 0.05$ as compared to the values obtained in resting control ganglia.

† $p < 0.05$ as compared to the values obtained in ganglia stimulated for 4 min at 60/sec.

TABLE XV

The effects of preganglionic stimulation at 20/sec and hemicholinium no. 3 (2 mg/Kg) on the number and distribution of dark-core vesicles in preganglionic nerve endings of sympathetic ganglia.

	Number of Dark-Core Vesicles per μ^2		
Experimental Conditions	Total	Synaptic[1]	Non-synaptic
a. Resting controls	5.5 ± 0.54	2.65 ± 0.77	5.90 ± 0.6
b. 5 min stimulation	6.45 ± 0.77	3.2 ± 1.00	6.75 ± 0.81
c. HC-3 and 5 min rest	4.55 ± 0.42	3.53 ± 0.68	4.70 ± 0.44
d. 5 min stimulation + HC-3	6.76 ± 0.78	4.18 ± 0.96	7.00 ± 0.80
e. 30 min stimulation	3.10 ± 0.39*	2.18 ± 0.86	3.20 ± 0.42*
f. HC-3 and rest for 35 min	4.50 ± 0.49	3.90 ± 1.00	4.58 ± 0.53
g. 30 min stimulation + HC-3	2.16 ± 0.40*	1.08 ± 0.49	2.15 ± 0.39*

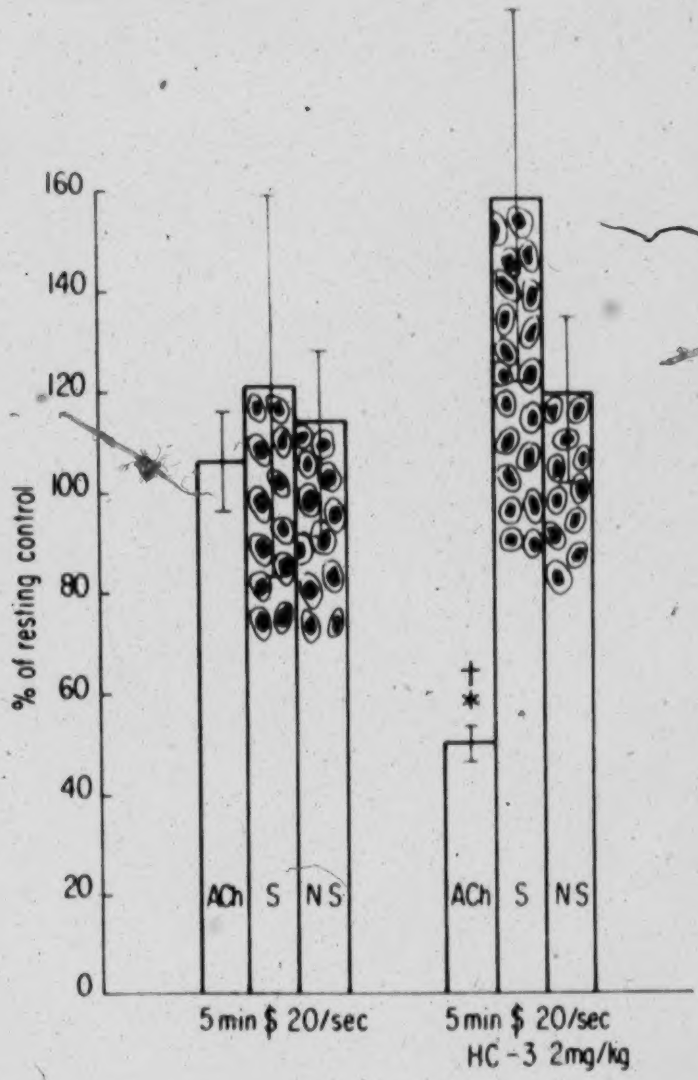
---

[1] Within a zone of 2000 Å from the synaptic cleft.

The values represent mean ± S.E. of 40 nerve endings taken from 5 to 7 ganglia in each case.

* $p < 0.05$ as compared to the values obtained in resting control ganglia.

Fig. 23. Effect of HC-3 (2 mg/Kg) and stimulation at 20/sec on the density of dark-core vesicles in different regions of nerve endings.



The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case.

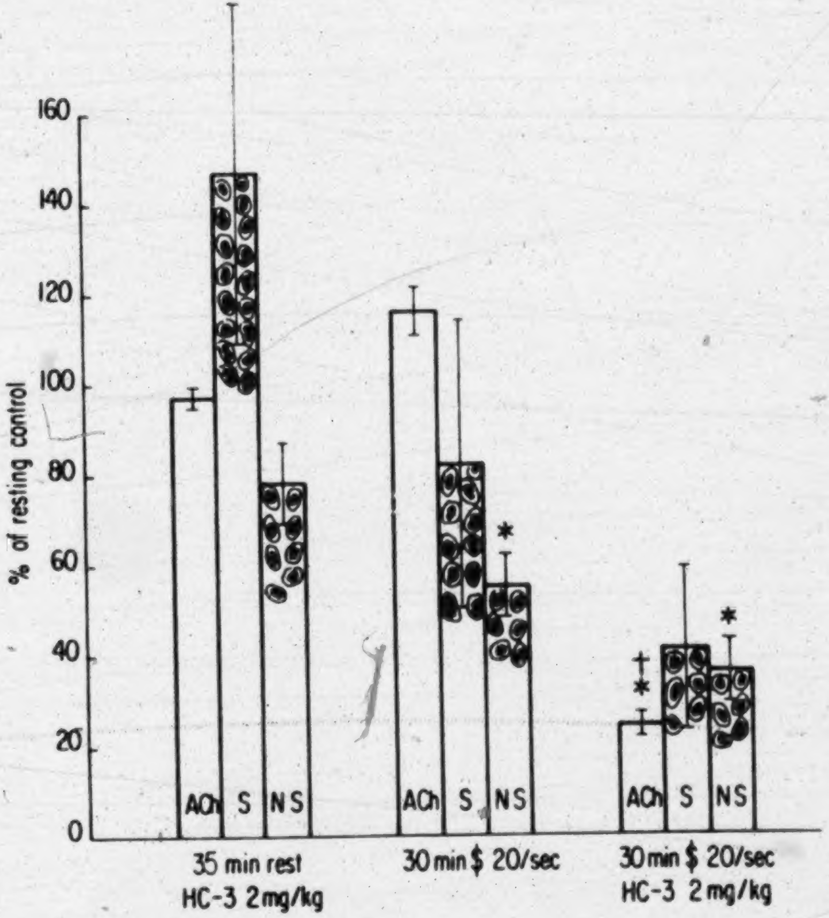
S represents the number of dark-core vesicles in synaptic zone.

NS represents the number of dark-core vesicles in non-synaptic zone.

* $p < 0.05$ as compared to the values obtained in unstimulated resting ganglia.

+ $p < 0.05$ as compared to the values obtained in ganglia stimulated for 5 min at 20/sec in absence of HC-3.

Fig. 24. Influence of preganglionic stimulation at 20/sec and hemicholinium no. 3 (2 mg/Kg) on the distribution of dark-core vesicles in different regions of nerve endings.



The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case.

S represents dark-core vesicles in synaptic zone.

NS represents dark-core vesicles in non-synaptic zone.

* $p < 0.05$ as compared to the values obtained in unstimulated resting control ganglia.

† $p < 0.05$ as compared to the values obtained in ganglia resting for 35 min pretreated with HC-3.

TABLE XVI

Effects of preganglionic stimulation at 20/sec and hemicholinium no. 3 on the density of agranular and dark-core vesicles in the preganglionic nerve endings of superior cervical sympathetic ganglia of the cat.

Experimental Conditions	Number of Vesicles per μ^2 of Nerve Ending Area	
	Agranular	Dark-Core
a. Resting controls	141 ± 6.5	5.50 ± 0.54
b. 5 min stimulation	141 ± 10.6	6.45 ± 0.77
c. HC-3 and 5 min rest	136 ± 8.9	4.55 ± 0.42
d. 5 min stimulation and HC-3	133 ± 9.2	6.76 ± 0.78
e. 30 min stimulation	107 ± 7.8*	3.10 ± 0.39*
f. HC-3 and rest for 35 min	150 ± 9.8	4.50 ± 0.49
g. 30 min stimulation and HC-3	65 ± 5.9*†	2.16 ± 0.40*

The values are mean ± S.E. of 40 nerve endings representing 5 to 7 ganglia in each case.

* $p < 0.05$ as compared to the values obtained in resting control ganglia.

† $p < 0.05$ as compared to the values obtained in resting ganglia exposed to HC-3 for 35 min.

DISCUSSION

A. ACh Turnover During Activity in Superior Cervical Ganglion

Preganglionic stimulation under physiological conditions causes little or no change in ganglionic ACh content (Birks and MacIntosh, 1961). However, there is a brisk turnover of ACh in such ganglia (as shown by these workers in ganglia stimulated at 20/sec) and they release about 8 - 10% of their resting content every minute. Obviously under these circumstances, the rate of ACh discharge matches the rate of ACh synthesis. Rosenblueth *et al.* (1939) have previously reported (based on single experimental observations) that the ACh content of ganglia first decreases and then increases during prolonged preganglionic stimulation at 60/sec. While preganglionic stimulation at 60/sec is grossly unphysiological the results obtained by these workers were of sufficient interest to warrant further research. Our findings represent a much more detailed study of these phenomena and explain many aspects of storage, synthesis and release of ACh in the superior cervical ganglion.

That preganglionic stimulation at 60/sec can reduce ACh content of ganglia was confirmed. Our data suggest that ganglia release more than 30% of their ACh content within 2 min when stimulated at this frequency, since some of the ACh liberated during this period of stimulation must have been replaced by resynthesis. The data obtained by Birks and MacIntosh (1961) suggest that more ACh can be released during the first 5 min of stimulation at 64/sec than at 16/sec; thereafter, however, the rate of ACh release declines such that during later periods of stimulation the ACh liberated per min is the same at both frequencies of stimulation. Since we have shown that during continuous stimulation at 60/sec the ganglia recover their ACh stores lost during the initial period of stimulation, it is evident that the arrival of impulses at the nerve endings, besides triggering the release of ACh, also stimulates its synthesis. Evidently the accelerated rate of ACh synthesis at 60/sec is probably not enough to replace the high initial output of ACh. At lower frequency of stimulation, however, the rate of ACh synthesis can be increased sufficiently to replace the more modest amount of ACh released during the brief period of stimulation. The above differences may explain why we were unable to demonstrate a reduction in the ACh content of ganglia stimulated for 2 min and 8 min at 20/sec.

MacIntosh (1963) suggested that there may be a delay in the onset of maximal rate of ACh synthesis upon nerve stimulation. In this connection he reported that stimulation at 20/sec for 5 min decreased the ACh content of ganglia by about 13%. However, if there is a significant delay in the onset of accelerated ACh synthesis induced by nerve stimulation the time lag must be less than 2 min because we were unable to demonstrate a decrease in ACh content when ganglia were stimulated at 20/sec for 2 min. Evidently in this case the amount of ACh liberated could be completely replaced by resynthesis by the end of the stimulus period.

The synthesis of ACh appears to continue at an accelerated rate for at least 10 min after the cessation of nerve stimulation. The amount (approximately 90 ng) of ACh lost during the preganglionic stimulation at 60/sec for 4 min was restored within 2 min during the subsequent rest period; but the ACh levels continued to rise and reached maximal levels of 130% of control after 10 min of rest. During the initial 2 min of rest these ganglia synthesized at least 45 ng/min; this rate is considerably higher than 29 ng/min observed by Birks and MacIntosh (1961) in plasma perfused ganglia stimulated at 20/sec. It is conceivable that the actual rate of synthesis in plasma perfused ganglia, stimulated at 20/sec, may have been higher than measured by these workers. Some of the deficite may be due to an incomplete recovery of ACh because some of the synthesized ACh may have passed into adjacent cellular structures and failed to enter the perfusion stream. Our findings also emphasize the necessity for the rapid removal of nerve tissue and the immediate inactivation of choline acetyltransferase when studying tissue levels of ACh.

Our results are in general agreement with the proposals of Potter *et al.* (1968) who have suggested that ACh synthesis is regulated by mass action laws. According to this view the synthesis of normal amounts of ACh depend upon the equilibrium position of the enzyme choline acetyltransferase with all four substrates, that is, choline, acetyl coenzyme A, ACh and coenzyme A. For example, stimulation at 60/sec reduced the ACh content and somewhat later increased the ganglionic choline levels; both of these events would favour a marked increase in the rate of ACh synthesis. Similarly such changes in the concentration of choline and ACh may also explain the rapid restoration in the ACh content during initial resting period and the sustained rebound increase in the ACh levels observed after 10 and 20 min of rest. The main driving force for the restoration of the ACh stores could be due to a fall in the concentration of ACh in the vicinity of choline acetyltransferase, and the accumulation of extra amounts of ACh may be largely the results of excessive uptake of choline. The sustained rebound increase may be explained if one assumes that the storage capacity of ACh, under these conditions, is also increased. Studies on minced rat brain indicate that the level of bound ACh is significantly increased in the presence of excess of choline content in the incubation medium (Bhatnager and MacIntosh, 1967).

A combination of many other factors may be responsible for maintaining the levels of ACh stores. For example, the concentration of sodium, an ion whose intracellular concentration increases with nerve depolarization, may regulate the activity of choline acetyltransferase and thus the levels of ACh. *In vitro* studies indicate that sodium ion concentration can stimulate the activity of the enzyme (McCaman and Hunt, 1965). Birks (1963) has suggested that increased sodium ion concentration, following nerve stimulation, may promote the transport of metabolic substrates to the enzyme involved in ACh formation. However, little is known about the relative abundance or rate limiting concentrations of acetylcoA or choline available for ACh synthesis *in vivo*. Product feedback inhibition may regulate the ACh levels has also been suggested (Kaita and Goldberg, 1969). To support such a hypothesis, Kaita and Goldberg have assumed that cholineacetyltransferase enzyme is bound within the vesicles, where ACh is synthesized and stored. However, evidence indicates that this enzyme may be localized in the cytoplasm rather than being bound within synaptic vesicles (Fonnum, 1967; Potter *et al.*, 1968).

In order to explain the constancy of ganglionic content of ACh under most circumstances, MacIntosh (1963) concluded that the amount of ACh in sympathetic ganglion is determined not merely by the balance between the processes favouring synthesis and the processes favouring release, but primarily by the number of sites available for the binding of ACh. According to this proposal when the binding sites are saturated, the excess of ACh formed is immediately destroyed by intracellular cholinesterase. Although most of our findings are in agreement with this proposal, the hypothesis cannot explain the rebound increase in ACh content which was observed in absence of anticholinesterase drug. Our results suggest that either additional binding sites can be rapidly formed or that the existing storage capacity is not normally saturable. Mechanisms apparently do exist to maintain ACh at relatively constant levels under normal conditions, and to place a ceiling on the content of ACh which can accumulate in the nerve tissue.

B. Readily Releasable Fraction of ACh in Superior Cervical Sympathetic Ganglia of Cat

Birks and MacIntosh (1961) have proposed that a small fraction of the ACh stores in a ganglion may be present in the form which can be immediately released by nerve impulses. Our results are consistent with this concept of a readily releasable pool and provides further information on the amount of ACh which is readily available for liberation.

The ability of stimulation at 60/sec to decrease the ACh content by 30% can be interpreted to mean that more than one-third of total ACh stores of a rested ganglion can be mobilized for release within 2 min; for one must assume that some of the ACh liberated during this stimulation period was replaced by resynthesis. This compares with a much lower estimate of 50 ng to be present in this pool, suggested by Birks and MacIntosh (1961). However, in order to make an accurate estimate of the size of this pool on the basis of a reduction in the ACh content, one would have to know not only the amount of preformed ACh which was mobilized into a releasable form, but also the quantity of the recently synthesized ACh retained by the ganglion during the 2 min period of stimulation.

When HC-3 (1 mg/Kg) was used to prevent ACh synthesis, the time-course studies of this dose of HC-3 in stimulated (20/sec) ganglia revealed that about 50% of ACh may be in the readily releasable form and the rest is only slowly converted into the releasable fraction. Although 1 mg/Kg HC-3 appeared to have blocked the ACh synthesis, there was a delay of about 5 min before any inhibition was obvious. When the dose of HC-3 was increased to 2 mg/Kg, the drug appeared to block the ACh synthesis immeidately and a reduction of 50% in ACh was observed within the first 5 min. Increasing the dose of HC-3 to 4 mg/Kg did not produce any greater depletion in ACh content during the first 5 min.

If one assumes that ACh synthesis is virtually prevented during the 30 min period of stimulation (in the presence of 2 mg/Kg HC-3), it then appears that about 50% of the total ACh stores can be rapidly mobilized for release, whereas the remainder is only slowly converted to a releasable form. The estimate of 50% agrees with our predictions based on the ability of preganglionic stimulation at 60/sec to reduce ganglionic ACh by 30% within 2 min.

C. Choline Metabolism

Ample evidence is now available to indicate that the ganglion needs a supply of extracellular choline for the maintenance of its ACh stores and output during prolonged activity (Brown and Feldberg, 1936; Birks and MacIntosh, 1961). Similarly, labelling experiments have shown that some of this choline can be incorporated into ACh (Friesen *et al.*, 1965; Potter, 1968; Collier and Lang, 1969). The experiments of Collier and MacIntosh (1969) have demonstrated that preganglionic stimulation increases the uptake of radioactive choline from the perfusion fluid and its incorporation into ACh. Our results (Fig. 1 and 2) provide similar conclusions that preganglionic stimulation can transiently increase the ganglionic content of choline. The evidence obtained by Friesen *et al.* (1967) suggests that less than one-third of the total choline content of ganglia resides in the presynaptic nerve terminals. Therefore, while the increases (25 - 30%) in choline levels observed in the present investigation were small, they may represent large changes in choline concentration in the preganglionic nerve endings provided that this is the site of choline accumulation. In this connection we have demonstrated that HC-3 is capable of preventing not only the recovery of ACh stores, but also the subsequent increases in both ACh and choline levels induced by 4 min stimulation and 10 min of rest (Fig. 3). These latter results are in accord with the hypothesis that HC-3 inhibits ACh synthesis by blocking the transport of extracellular choline to the sites of acetylation (MacIntosh *et al.*, 1958).

The fast recovery of ACh and the temporary increase in choline content after 4 min of stimulation at 60/sec followed by 10 min of rest (Fig. 2) demonstrates that the preganglionic nerve endings may efficiently capture the extracellular choline and concentrate within the nerve terminals. However this accumulation of extra choline was only transient and the excess is either quickly removed or utilized for ACh synthesis. Although Potter's (1968) findings also suggest that the isolated nerve endings from brain can concentrate choline from the surrounding medium, our data does not provide evidence that the accumulation of choline is against the electrochemical gradient. The results, however, suggest that the choline transport system which subserves ACh synthesis may be unique and more sensitive to HC-3 inhibition than the general choline transport system which subserves phospholipid synthesis. Other lines of evidence tend to support the above hypothesis. For example, doses of HC-3 which impair ACh synthesis do not reduce the incorporation of choline into phospholipids or their precursers (Collier and Lang, 1969; Gomez *et al.*, 1970). In addition a number of studies (Martin, 1968; Chang and Lee, 1970; Cooke and Robinson, 1971) show that blockade of choline transport in other tissues generally require 5 to 20 times more HC-3 than the concentration which can effectively deplete ACh in sympathetic ganglia (Birks and MacIntosh, 1961; Matthews, 1966).

D. Mode of Action of Hemicholinium No. 3 to Prevent Synthesis of Acetylcholine in Sympathetic Ganglia of the Cat

The action of HC-3 to inhibit the ACh synthesis has been attributed to a competitive inhibition of choline transport across the cell membrane (Birks and MacIntosh, 1961; Hodgkin and Martin, 1965; Marshbanks, 1968; Collier and MacIntosh, 1969; Diamond and Milfay, 1972). In this connection we have demonstrated that HC-3 is capable of preventing not only the recovery of ACh stores, but also the subsequent increase in both ACh and choline levels induced by 4 min stimulation at 60/sec followed by 10 min of rest period.

During the course of our investigation on the effects of HC-3 (1 mg/Kg) on ganglionic ACh content it was found that while the onset and magnitude of transmission failure were related to the frequency of stimulation (Fig. 5), these effects could not be correlated with the percent reduction in ACh stores (Fig. 7). Ganglia stimulated for 30 min at 2 and 5/sec lost approximately 40% of their ACh content, but the contractile response of nictitating membrane was not appreciably altered. In contrast, evidence of marked transmission failure was evident by the end of 30 min in ganglia stimulated at 10 or 20/sec, in spite of the fact that the depletion, 50% of ACh, was comparable to that seen in ganglia stimulated at 2 and 5 pulses per sec. The above results can perhaps be best explained in terms of the quantity of ACh in the readily releasable pool. It can be argued that at low frequency of stimulation there was sufficient ACh in the readily releasable form to maintain transmission whereas, in ganglia stimulated at 10 or 20/sec, the onset and progressive decline in the membrane response was due to a gradual decrease in the quantity of ACh available for immediate release. The data also suggest that nictitating membrane response is an unreliable index of the inhibiting effect of HC-3 on ACh synthesis.

The time-course effects of HC-3 (1 mg/Kg) on ACh content of ganglia, stimulated at 20/sec, suggests that there is a delay of about 5 min in the onset of action of this drug (Fig. 7). At a dose of 2 mg/Kg HC-3 the most rapid decline of 50% in ACh stores occurred within the first 5 min (Fig. 8) and thereafter the ACh content decreased more slowly. These findings suggest that 2 mg/Kg dose of HC-3 is an optimal dose and is capable of immediately blocking ACh synthesis completely provided that the drug is administered prior to the onset of preganglionic stimulation. The data also suggest that about 50% of the ganglionic ACh stores can be readily mobilized for release and the rest is only slowly available for release.

A comparison of above results, in presence of 2 mg/Kg HC-3, with those obtained in plasma perfused ganglia (in absence of HC-3) by Birks and MacIntosh (1961) revealed that the initial minute (53 ng) and volley outputs (44 μμg) of ganglia exposed to HC-3 are greater than that observed in plasma perfused ganglia. One can conclude from these results that HC-3 may increase the initial volley output of ACh or alternatively, the initial volley output of blood perfused ganglia may be somewhat greater than that which can be achieved in plasma perfused ganglia. However, the data obtained with 4 mg/Kg HC-3 (Table V) contradicts the hypothesis that HC-3 can increase the initial release in ACh.

The failure of the 4 mg/Kg dose of HC-3 to produce the same or greater depletion of ACh stores than that obtained with 2 mg/Kg dose is difficult to explain. It was thought that HC-3, by blocking the uptake of choline by tissue or perhaps by blocking its renal excretion, may drastically raise plasma choline concentrations. This increase in plasma concentrations may in turn antagonize the effects of HC-3. While a 4 mg/Kg dose does increase plasma choline levels by 50% (Table VIII), this increment is probably too small to exert a significant antagonistic effect. Birks and MacIntosh (1961) have shown that about 1000:1 molar ratio of choline:HC-3 is required to completely antagonize the effect of HC-3 in sympathetic ganglia. MacIntosh (1963) reported that major pelvic and abdominal surgery or cortisone administration reduced plasma choline levels. In this regard our data has demonstrated that surgical procedures involved in exposing ganglia do not constitute a sufficient stress to significantly lower the concentration of plasma choline.

Another possibility is that the large doses of HC-3 may directly impair ACh release (Hart and Long, 1965; Chiang and Leaders, 1967) thereby decreasing the need for ACh synthesis which in turn would tend to nullify the ACh depleting action of the drug. However, the data obtained by Matthews (1966) does not provide any evidence that HC-3 decreases ACh release from sympathetic ganglia, even when large concentrations of the drug are used.

Results of our investigations on choline content (Table VII) suggest that HC-3 is capable of depleting the ACh content of ganglia without decreasing the choline levels in most cases. The data leads to the conclusion that HC-3 not only blocks the uptake of choline (geared to ACh synthesis) from extracellular fluid but may also inhibit the utilization of choline within the nerve terminals. Studies by

Friesen *et al.* (1967) suggest the preganglionic nerve endings may contain 60 to 80 ng of choline; this represents about 1/3 of the total choline content of ganglion. Therefore, if HC-3 only blocked the uptake of choline presynaptically, one would expect a rapid 33% reduction of choline prior to the onset of ACh depletion. Alternatively, perhaps most of the choline stored in the presynaptic nerve terminals is not normally available for ACh synthesis.

Ample evidence is available which suggests that HC-3 can be taken up into cholinergic nerve terminals (Sellinger *et al.*, 1969; Csillik *et al.*, 1970; Slater and Stonier, 1971; Collier, 1973) and may have an action at an intracellular site. The effect of HC-3 in depressing ACh turnover in perfused ganglia wears off only very slowly and requires much larger amounts of choline (molar ratio of choline:HC-3 = 1000:1) in the perfusion fluid to antagonize the effects of HC-3 (Birks and MacIntosh, 1961; MacIntosh, 1963). If HC-3 were acting only on the outside of presynaptic membrane, to block choline transport, we would expect its effect to be rapidly reversible. That HC-3 can be acetylated *in vitro* by choline acetyltransferase, has been suggested recently (Rodriguez de Lores Arnaiz *et al.*, 1970; Hemsworth, 1971). These findings indicate that acetylated HC-3 may act as a false transmitter. However, more recent work of Collier (1973) indicates that no such acetylated HC-3 is released in sympathetic ganglia during preganglionic stimulation. It is evident that HC-3 can be accumulated by the nervous tissue (Sellinger *et al.*, 1969; Rodriguez de Lores Arnaiz *et al.*, 1970; Collier, 1973) but whether or not it has any intracellular action still remains to be determined.

Our results suggest that HC-3 may compete with choline for transport to the acetylation site not only on the surface membrane, as suggested by MacIntosh (1963), but also within the nerve terminals. For example, if ACh is synthesized within the agranular vesicles, HC-3 may compete with choline at the vesicle membrane site. Collier (1973) has suggested that ACh may be synthesized within the cytoplasm and then transported to the vesicles for storage and release. He further suggested that HC-3 may compete with the transport of this cytoplasmic ACh to the synaptic vesicles. This may be true in the light of the evidence which indicates a cytoplasmic localization of choline acetyltransferase enzyme (Fonnum, 1967; Potter *et al.*, 1968). However, no direct evidence is available which can support such a hypothesis. Also it is difficult to envisage how the cytoplasmic ACh is protected from hydrolysis by acetylcholinesterases and how ACh subsequently gets into the vesicles.

That HC-3 influences arterial blood pressure, was a significant observation. While the 2 mg/Kg dose only causes a transient fall (~40%), 4 mg/Kg HC-3 produces not only an immediate decrease (~52%), but also a long-lasting reduction (~32%) in the blood pressure. The probability that 4 mg/Kg dose of HC-3 may produce a persistent blockade of postsynaptic ACh receptors was eliminated. The arterial injection of submaximal dose of ACh produced the same nictitating membrane response before and after the treatment with HC-3. Further research will be required to determine the mechanism of this action of HC-3.

Parducz *et al.* (1971) have used HC-3 concentrations as high as 10 mg/Kg in the intact cats. Our results indicate that 2 mg/Kg is an optimal dose of HC-3 and a further increase in the HC-3 dose can produce a sustained reduction in the blood pressure and may seriously impair the blood perfusion of the tissue in intact animals. Therefore, one must seriously question the validity of the results obtained at doses above 2 mg/Kg.

E. 1. Agranular Vesicles in Relation to the Storage and Release of ACh in the Preganglionic Nerve Terminals

The discovery that ACh is released in discrete quantal packages (Fatt and Katz, 1952; Del Castillo and Katz, 1954) and the subsequent findings that nerve endings contain numerous agranular vesicles lead to the hypothesis that agranular vesicles are involved in the storage and release of ACh (De Robertis and Bennett, 1955; Robertson, 1956). The vesicle hypothesis thus implies that the agranular vesicles contain multimolecular "quanta" of transmitter which is released in all or none fashion by a process of exocytosis (Hubbard, 1970; Smith, 1971).

Various indirect evidence support the above hypothesis and suggests that vesicles may recycle locally in a manner which allows each vesicle to package and release quanta of transmitter repeatedly (Bittner and Kennedy, 1970; Heuser and Reese, 1973). Ceccarelli *et al.* (1972) have shown that the frog nerve-muscle preparation maintains its population of agranular vesicles even after 4 hours of stimulation at 2/sec. When horse radish peroxidase was added to the medium and the preparation was stimulated for 6 - 8 hrs, a large number of vesicles were found to contain the peroxidase. These investigators concluded that their evidence was consistent with the concept that vesicles release ACh by exocytosis and that the vesicles are reutilized.

A number of other attempts have been made to test the vesicle hypothesis. Thus some authors have reported decreased number of synaptic vesicles during increased transmitter release (De Robertis and Vaz Ferreira, 1957; Birks, 1971) while others have not observed any change (Birks, Huxley and Katz, 1960; Green, 1966). Increasing the external potassium concentration at the neuromuscular junction causes a decrease in the number of vesicles close to the presynaptic membrane (Hubbard and Kwanbunbumpen, 1968). More recently Perri *et al.* (1972) have shown that preganglionic nerve stimulation of rat superior cervical ganglia causes a decrease in number of vesicles which is more pronounced as the distance from the synaptic junction increases.

Hemicholinium no. 3, which inhibits ACh synthesis has also been used to test the vesicle hypothesis. Elmquist and Quastel (1965) stimulated rat phrenic nerve diaphragm in presence of 4 x 10^{-6} M HC-3. They found that prolonged activation (40 - 60 min) using either nerve stimulation or raised potassium ion concentration causes a progressive decrease of quantum size without evidence of any effect on the number of quanta released. When these investigators allowed their preparations to rest after initial rundown of quanta, the size of the quantum was found to recover. An examination of their results indicate that the dose of HC-3 used by these investigators did not completely inhibit ACh synthesis and may have partially allowed the refilling of vesicles. In addition their data suggest that vesicles may be capable of releasing ACh even though they do not contain their normal complement of transmitter.

More recently Sacchi and Perri (1973) have investigated these phenomena in isolated rat superior cervical ganglia. They used HC-3 in a concentration of 6 x 10^{-6} M and used a choline free bathing solution. Upon preganglionic stimulation at 10/sec there was a rapid onset of transmission failure which became quite marked after 5 - 6 min of stimulation. They concluded that the transmission failure was due to a decrease in number of quanta with little or no change in quantum size. Their results can be reconciled with those of Elmquist and Quastel if one assumes that they obtained immediate and complete block of ACh synthesis. As a consequence previously filled vesicles were competing with empty vesicles for release resulting in a reduction in the number of measurable quanta released, but no change in the quantum size.

The evidence presented thus far suggests that the agranular vesicles may be involved in storage and release of ACh but to our knowledge no attempt has been made to directly relate the ACh content with the number of agranular vesicles. In other words the actual ACh content was not determined by these workers. In our present study we have attempted not only to correlate the ultrastructural changes with the depletion in ACh content but also determined how readily *reversible* the induced structural changes are, and how this relates to the rates of recovery of the ACh content. In addition previous workers have used prolonged stimulation to cause alterations in the ultrastructure whereas, we have attempted to demonstrate a correlation between the ACh content and the number of agranular vesicles after a very short period of stimulation.

Before any physiological significance is attached to our data, the following assumptions must be made in the light of above presented evidence. (1) Under most physiological conditions agranular vesicles in the preganglionic nerve terminals are repeatedly utilized after releasing their transmitter quanta by exocytosis. (2) To be morphologically or functionally active, agranular vesicles do not have to contain their full complement of transmitter. (3) Vesicles normally store ACh only up to 60 - 70% of their total storage capacity.

The first two assumptions are based on the evidence presented above (Bittner and Kennedy, 1970; Ceccarelli *et al.*, 1972; Elmquist and Quastel, 1965). The third assumption is based on our data (Friesen and Khatter, 1971) which demonstrates a rebound increase in ACh content of ganglia after 20 min of rest following 4 min of stimulation at 60/sec.

In our previous work we have demonstrated that preganglionic stimulation at 20/sec for 5 min maintains the ACh stores of ganglia at control level. When compared with ultrastructure, the population of agranular vesicles were also found to be at control level under these conditions. Apparently at this frequency of stimulation, synthesis of ACh and reformation of vesicles can keep pace with release during a short period of stimulation. When the stimulation frequency was increased to 60/sec [which causes about a 30% decrease in ACh content (Fig. 1)] a significant (39%) reduction in the density of agranular vesicles also occurred within 4 min (Fig. 14). The discovery that such a short period of stimulation can cause a significant depletion in both ACh and the number of agranular vesicles is strong evidence in favour of the concept that vesicles are storage sites of ACh. A preliminary paper on this aspect has already been published (Friesen and Khatter, 1971). More recently Korneliussen *et al.* (1972) have found a similar reduction of 43% in agranular vesicles in phrenic nerve diaphragm preparation, stimulated at 50 hz for 5 min. In another experiment these workers increased the stimulation frequency to 100 hz and found that the reduction in number of vesicles can be produced within 20 sec. These results agree with our data, which demonstrate the rapidity with which these vesicles can be depleted if nerves are stimulated at high frequency.

At 60/sec stimulation for 4 min although the simultaneous reduction of ACh and the density of agranular vesicles strongly support the vesicle hypothesis, the two events may not be directly related. Since synthesis of ACh is greatly accelerated on stimulation at this frequency (Fig. 2), the rate of reformation of vesicles may not be compatable with this new rate of ACh synthesis. Alternatively some of the vesicles may be structurally damaged due to the unphysiologically high frequency of stimulation. Thus under these conditions some vesicles may store more ACh than others which have not depleted their ACh content.

The above view is further strengthened if one considers the situation where ganglia recover their lost ACh within 2 min of rest (Fig. 2). Under these conditions although ganglia recovered their ACh content, released during 4 min of stimulation at 60/sec, the number of agranular vesicles were still significantly lower than controls (Fig. 14). Similarly when the resting period was extended to 20 min, to cause a 30% rebound increase in ACh content, the population of the vesicles recovered only to the normal level and there was no increase in their number corresponding to the rebound increase in ACh. These data lead to two main conclusions. (1) There may be an incompatability between the rates of ACh synthesis and the reformation or reutilization of depleted vesicles under these unphysiological stimulation conditions. (2) The agranular vesicles do not normally store ACh to their full capacity and under these abnormal conditions may contain ACh up to their saturation level. Alternatively, the storage capacity of agranular vesicles is significantly increased under these conditions.

The reduction in the number of agranular vesicles in ganglia stimulated at 20/sec for 30 min can also be explained on the basis of the above hypothesis. Under these conditions it was found that, although, the ACh content was maintained at control level, the population of agranular vesicles was reduced by 24%. Perri *et al.* (1972) found a similar reduction (32%) of agranular vesicles in rat superior cervical ganglia, which was stimulated for 3.5 hrs at 0.5/sec followed by 30 min stimulation at 20/sec. The reduction in the number of vesicles during this prolonged period of stimulation may have occurred due to the damage of the vesicle membrane structure after repeated utilization. Alternatively, there may be an incompatability between the rate of ACh synthesis and the recycling of vesicles. Whatever the reason may be, the data suggest that the vesicles under these conditions store more than their usual content of ACh.

The data obtained in ganglia exposed to HC-3 also support the above hypothesis. When the animals were treated with 2 mg/Kg HC-3 5 min prior to the stimulation (20/sec), the ganglia released their 50% of ACh stores within 5 min. However, the number of agranular vesicles under these conditions still appeared to be at control level (Fig. 16).

As presented in the previous section (Fig. 8) this dose of HC-3 is capable of completely blocking ACh synthesis immediately. We have also demonstrated that in absence of HC-3, stimulation at 20/sec for 5 min maintains the vesicle population at control level (Fig. 16). These observations suggest that during 5 min of stimulation in presence of HC-3 (2 mg/Kg) a large number of the vesicles may remain completely empty after releasing their transmitter content. If these empty vesicles are still functional and have the same probability of exocytosis, the number of quanta released at any time will be greatly reduced. The observations made by Sacchi and Perri (1973) in rat ganglia support this view. These workers demonstrated that preganglionic stimulation of rat ganglia in presence of HC-3 or thiamine deprivation causes a reduction in number of quanta released without causing any change in the size of individual quantum. The hypothesis may also explain why the nictitating membrane response is greatly reduced under these conditions (Fig. 5) when the ganglia still contain about 50% of their normal ACh content.

A much larger depletion (54%) in the number of agranular vesicles was observed when the stimulation period was extended to 30 min in ganglia exposed to HC-3 (Fig. 18). Under these conditions ganglia also released about 75% of their transmitter stores. It is noteworthy that the depletion in the population of vesicles in absence of HC-3 was only of the order of 24%. The extra decrease in the number of vesicles may be due to the following factors: (1) A non-specific action of HC-3 on the vesicle membrane structure, (2) the reduction in the available choline for the formation of vesicle membrane structure in presence of HC-3. The latter possibility is supported by the fact that only under these conditions was a significant reduction (28%) in choline content of ganglia observed (Table VII). The data obtained by Párducz and Fehér (1970) also support this hypothesis. These investigators observed that when cat ganglia were perfused with choline-free Locke's solution and stimulated at 20/sec, most of the agranular vesicles in the nerve terminals were depleted. But no such depletion occurred when choline (10 mg/ml) was added in the perfusion fluid. The data obtained by Collier and Lang (1969) indicate that HC-3 does not influence significantly the incorporation of choline into phospholipids. However, the incorporation of choline into phosphorylcholine was found to be slightly reduced.

2. Mobilization of Agranular Vesicles During Nerve Stimulation

As stated before an increase in external potassium concentration causes a decrease in number of vesicles close to the presynaptic membrane (Hubbard and Kwanbunbumpen, 1968). On the other hand depolarizing currents applied to nerve terminal were found to increase the number of vesicles in this zone close to the presynaptic membrane (Landau and Kwanbunbumpen, 1968). Similarly high frequency stimulation has been shown to increase (Jones and Kwanbunbumpen, 1968; Hubbard, 1970) or decrease (Korneliussen, 1972) in the number of vesicles in the 1800 Å region near the synaptic cleft.

More recently Perri *et al.* (1972) found that in the preganglionic terminals of rat, the vesicle depletion during nerve stimulation is more pronounced as the distance from the synaptic junction increases. Somewhat similar observations were made in the present investigations. A significant depletion in agranular vesicles during 30 min stimulation at 20/sec (Table X) or 4 min stimulation at 60/sec (Fig. 15) occurred mainly in the region away from the synaptic zone (2000 Å region close to the presynaptic membrane). Prolonged stimulation (20/sec) in presence of HC-3 also produced the depletion of vesicles mainly in the non-synaptic region (Table X). Under either of the above conditions no statistically significant change in the number of vesicles was observed in the zone (2000 Å) near the synaptic cleft. On the assumption that the vesicles release ACh by exocytosis, the above data suggest that the agranular vesicles may be mobilized towards the synaptic cleft during (preganglionic) stimulation.

The decrease in number of vesicles in the preganglionic nerve endings caused by stimulation or stimulation combined with HC-3 demonstrates that vesicles may be involved in synaptic transmission and that vesicle supply may be inadequate to compensate for the vesicles lost during sustained stimulation. The fact that vesicles in non-synaptic region deplete more readily (Table IX and X) suggests that: (1) mechanisms may exist to maintain a high concentration of vesicles near the presynaptic membrane and cause efficient release of transmitter and that (2) during stimulation vesicles flow toward the specialized area of the presynaptic membrane.

As to the mechanism of transmitter release, some speculative remarks can be made. The electron microscopic observations provide strong evidence that the vesicles fuse with the presynaptic membrane (Couteaux and Pecot-Dechavassine, 1970; Nickel and Potter, 1970; Ceccarelli *et al.*, 1973) indicating a model for transmitter release by exocytosis. The reduced number of agranular vesicles, following stimulation, in our experiments fits this concept. However, the difference in membrane composition between synaptic vesicles and the presynaptic membrane [Whittaker (1970) in excitatory synaptic mechanisms, (Eds. P. Andersen and J.K.S. Jansen; Universitetsforloget, Oslo), p. 67] makes a definite fusion improbable. On the other hand if the fusion is only temporary, our results favour the reusage of vesicles. In this regard we determined the area of nerve endings before and after stimulation and stimulation combined with HC-3 (Table XI). Our results agree with those of Pysh and Willey (1972) who found that the areas of the nerve endings remain constant after their stimulation or stimulation combined with HC-3. However, a separate machinery may be available to synthesize new vesicles to replace those lost during continuous use for transmitter release. Suggestions have been made that mitochondria may be involved in the formation of new vesicles (Dyatchkova *et al.*, 1962; Hubbard and Kwanbunbumpen, 1968). Our electronmicroscopic observations show similar evidence of swellings of mitochondria during stimulation. The mitochondria observed in unstimulated nerve endings were generally normal in appearance. Many other sites of synthesis of these vesicles have been hypothesized, e.g., endoplasmic reticulum (Palay, 1958), Golgi complex (Van Breemen, Anderson and Reger, 1958) and neurotubules (De Robertis, 1967). More recently the formation of vesicles directly from the terminal membrane has been suggested (Ceccarelli *et al.*, 1972). The evidence thus far presented suggests that during the transmitter release the original vesicles return to the cytoplasm and there is no immediate permanent loss of vesicles. The depletion of vesicles we observed during 60/sec stimulation for 4 min followed by 2 min of rest or prolonged stimulation at 20/sec (no HC-3) may simply indicate an incompatability between the rate of reformation or reutilization of vesicles and the rate of synthesis of ACh.

As to the significance of general procedure, sufficient attempts were made to handle appropriate controls and experimental preparations identically except for the omission of stimulation or HC-3 treatment in the former. The procedures of sampling and obtaining quantitative data are regarded sufficient to exclude any bias. It is true that not all the nerve endings obtained from stimulated preparations appeared to have vesicles depleted. However, a much larger number of nerve endings (a total of 40 nerve endings taken from 5 to 7 ganglia in each case) were used in the present investigation to determine the significance of our findings.

Controversy still exists about the effects of fixative mixture used. Thus Birks (1971) has reported that the preservation of agranular vesicles, obtained in the superior cervical ganglion, is improved if the usual sodium phosphate buffer was replaced by Hepes buffer containing a high concentration (110 mM) of magnesium chloride, in the 3.5% gluteraldehyde fixative. When a similar fixative containing 2% gluteraldehyde was used by Cochrane *et al.* (1972) for the excitatory nerve-muscle synapse of the locust, no such improvement in the ultrastructure was observed. However, when they raised the glutaraldehyde concentration of magnesium fixative to 3.5%, results similar to those of Birks were obtained. Fixative containing zero calcium did not cause any improvement in the ultrastructure of the nerve endings. Atwood *et al.* (1972) used crayfish nerve-muscle preparation for their investigations. These workers observed that the treatment of the resting preparation with magnesium fixative results in a high vesicle count as reported by Birks (1971). However the qualitative changes in the number of vesicles after stimulation were found to be same whether or not magnesium was added to the fixative. It is, therefore, difficult to interpret the significance of addition of magnesium chloride in the fixative mixture. Further work would certainly be required to clarify these controversial observations.

F. Influence of Experimental Conditons, Which Alter Ganglionic ACh Stores on the Density of Dark-Core Vesicles in the Preganglionic Nerve Terminals

Large dark-core vesicles, often seen in cholinergic nerve endings, have been suggested to store catecholamines similar to those in the adrenergic nerve endings (Wolfe *et al.*, 1962; Richardson, 1964; Clementi, 1966). Other workers (Grillo and Palay, 1962; Elfvin, 1963) compare them to the vesicles concerned with neurosecretion which are found in large quantities in hypothalamus-pituotary system (Ledris, 1965). More recently it has been suggested that they may store some trophic materials (Atwood *et al.*, 1971). If these dark-core vesicles are involved directly or indirectly in the synaptic transmission, the preganglionic stimulation should deplete their unknown content similar to the agranular vesicles which store ACh.

Preganglionic stimulation at 60/sec for 4 min causes about a 30% reduction in the ACh content, a 46% decrease in the number of agranular vesicles (Fig. 14) and a 34% reduction in the population of dark-core vesicles (Table XIV). The recovery in the density of dark-core vesicles in the depleted nerve endings, similar to those in ACh content and the population of agranular vesicles (Fig. 14), was also observed when these ganglia were allowed a 20 min rest after initial 4 min of stimulation at 60/sec (Fig. 22). Although significant changes, in the population of dark-core vesicles, were demonstrated only in non-synaptic region, similar changes in the population of these vesicles in the synaptic region (a zone of 2000 Å from the synaptic cleft) also seemed to occur. However, because of the high standard errors of up to 30% even large alterations in number of dark-core vesicles in the synaptic zone could not be demonstrated as statistically significant. Since most of these dark-core vesicles in resting control nerve endings are generally localized in the non-synaptic region, the data demonstrates that dark-core vesicles flow from the non-synaptic to the synaptic region and deplete their unknown content during preganglionic stimulation. The recovery in the population during 20 min of rest following the initial depletion during 4 min stimulation at 60/sec suggests that similar to agranular vesicles, dark-core vesicles are reformed or recycled after depletion of their stored content.

Stimulation of ganglia at 20/sec for 30 min, pretreated with HC-3 (2 mg/Kg), which depletes about 75% of the ACh also caused a significant reduction (64%) in the number of dark-core vesicles mainly in the non-synaptic region (Fig. 24). There was a large variation in their numbers particularly in the synaptic zone, and even the changes of 59% could not be demonstrated as statistically significant. Hemicholinium no. 3, in absence of stimulation, did not produce any statistically significant alteration in their number in any of the regions determined. This again may be due to the large variation in their density observed particularly in the synaptic zone.

Preganglionic stimulation at 20/sec, for 30 min, in absence of HC-3 caused a significant reduction in the number of dark-core vesicles, which occurred mainly in the non-synaptic region. However, under such conditions, the levels of ACh stores were not significantly altered. Also, when ganglia were allowed to rest, for 20 min, after initial 4 min of stimulation at 60/sec, there was no increase in the number of dark-core vesicles corresponding to a 30% rebound increase in ACh content. Similarly when ganglia were stimulated for 5 min, at 20/sec, in the presence of HC-3 the ganglia lost about 50% of the ACh content but no significant reduction in the density of dark-core vesicles could be demonstrated. These latter data suggest that there is no direct correlation between ACh content of ganglia and the number of dark-core vesicles in the preganglionic nerve terminals.

The functional role of the large dark-core vesicles is still controversial because they are seen both in monoaminergic and non-monoaminergic neurons, Furthermore, in several studies no obvious changes in number and appearance of these vesicles were found after reserpine treatment known to reduce amine levels (Taxi, 1965; Bondareff, 1965, Clementi *et al.*, 1966). In general our results show that the same conditions which deplete agranular vesicles also cause a reduction in the number of these dark-core vesicles (Tables XIV and XVI). From these data one can be lead to interpret that these dark-core vesicles may even store ACh. However, no other evidence is available which may suggest such conclusions.

The above data thus demonstrate that the dark-core vesicles in preganglionic nerve terminals flow from non-synaptic to synaptic region, deplete their unknown content and even are reutilized or recycled during preganglionic stimulation.

SUMMARY

The following are the general conclusions drawn from the data presented in this thesis. These conclusions are based on the assumption that the transmitter ACh is stored and released in association with the agranular vesicles.

(1) Stimulation at 60/sec reduced the ACh content and somewhat later increased the ganglionic choline levels; both of these events would favour a marked increase in the rate of ACh synthesis. These results are compatable with the hypothesis (Potter *et al*, 1968) that the ACh synthesis in the preganglionic nerve terminals is regulated by mass action laws.

(2) When ganglia were allowed to rest after 4 min of stimulation at 60/sec a rebound increase of 130% in ACh content was observed. However, no parallel increase in the number of agranular vesicles was observed under these conditions. Preliminary experiments indicate that the extra 30% ACh can be released by nerve stimulation. Thus if ACh is stored in and released from agranular vesicles, the data indicate that vesicles, under physiological conditions, do not store ACh to their saturation capacity.

(3) When ganglia are pretreated with HC-3 (2 mg/Kg) and stimulated at 20/sec, a 50% reduction in the content of ACh occurs within 5 min. The data lead to the conclusion that 50% of total ACh can be rapidly mobilized for release and may represent readily releasable fraction.

(4) The data obtained on choline content (Table VII) suggest that HC-3 is capable of depleting the ACh content of ganglia without decreasing

the choline levels in most cases. This suggests that HC-3 may not only compete with choline for transport at the external surface of the membrane but may also inhibit the utilization of choline within the nerve terminals.

(5) Stimulation of ganglia, pretreated with HC-3, for 5 min at 20/sec did not change the number of vesicles but decreased the ACh content by 50%. This suggests that if agranular vesicles release ACh by exocytosis, the vesicles are repeatedly utilized for storage and release of ACh. In addition these results indicate that vesicles may be capable of storing less than their normal quota of ACh.

(6) The observations that the agranular vesicles in the non-synaptic region tend to deplete more readily (Table IX and X) suggest that:

(a) a mechanism may exist to maintain a high concentration of vesicles in the synaptic zone to ensure efficient release of transmitter

(b) during stimulation vesicles are mobilized toward the synaptic cleft.

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